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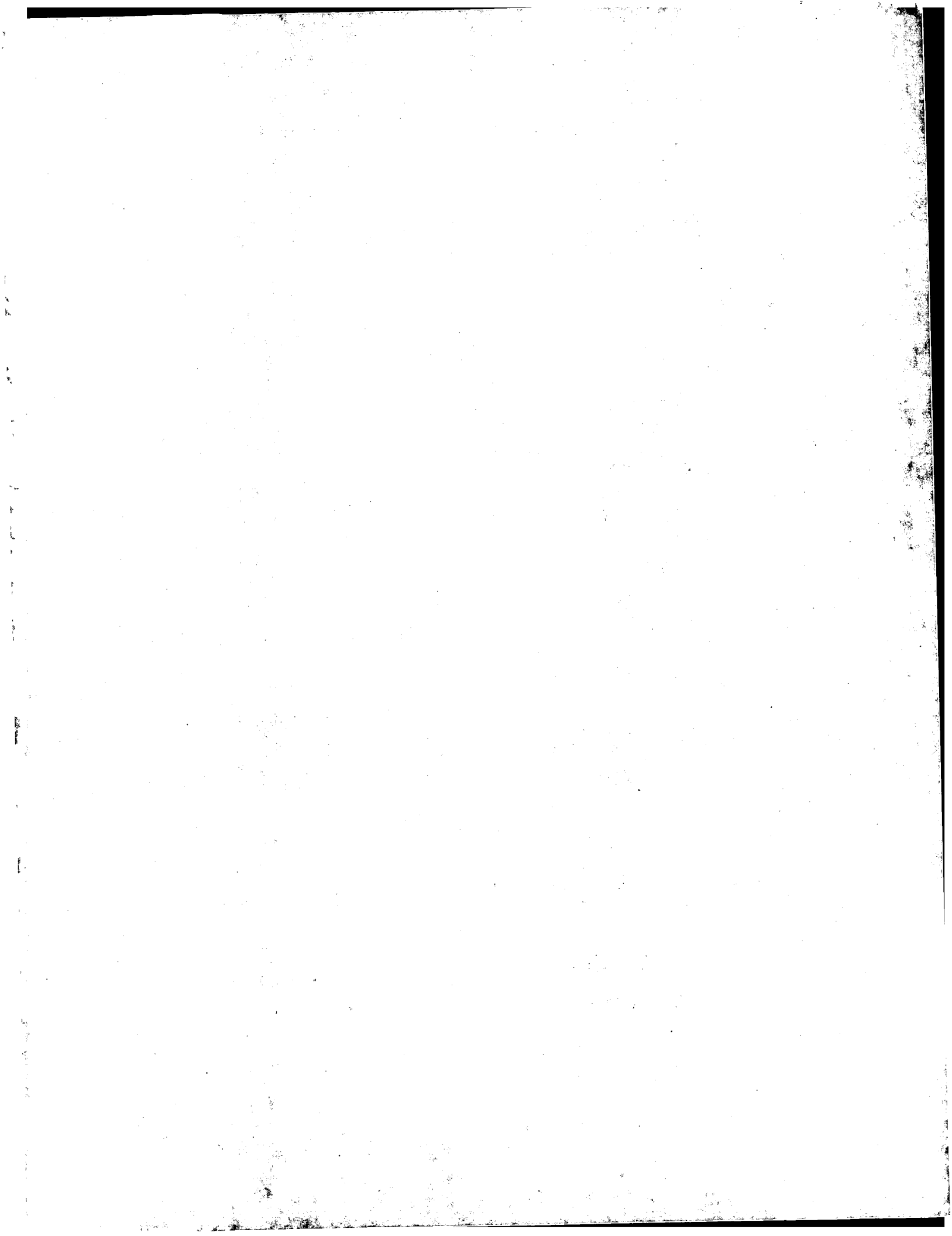
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(54) Title: COMPOSITE PLASMIDS FOR AMINO ACID SYNTHESIS (57) Abstract Composite plasmids containing multiple genes in transcriptional units. These composite plasmids are useful for the production of amino acids, particularly aromatic amino acids.		

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5

"Composite Plasmids for Amino Acid Synthesis"

10 BACKGROUND OF THE INVENTION

1. Field of the Invention

15 Described are methods for the production of composite plasmids containing multiple genes that have been individually mutated then assembled into transcriptional units. These composite plasmids are useful in the production of amino acids and other metabolites and in screening for superior host cells.

20

2. Description of the Prior Art

25 Bacterial fermentations are extensively employed for the industrial production of both primary metabolites such as amino acids and secondary metabolites such as antibiotics. The production of these metabolites is an industrial process of great economic significance.

30 Naturally occurring microorganisms do not in general overproduce any metabolites since the relevant biosynthetic pathways are tightly regulated to avoid any waste of resources. The development of industrially useful production strains requires an extensive program of mutagenesis followed by selection of improved strains. Such a selection process may become extremely laborious in the later stages of strain development and

35

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even involve the screening of individual isolates by fermentation.

Recombinant DNA technology has greatly facilitated strain development since it is now possible to isolate the important genes in a biosynthetic pathway and to deregulate them in vitro. In addition, the cloning of genes onto multicopy plasmids is an efficient way of boosting the expression of a gene and hence the level of the relevant protein within the cell.

Many biosynthetic pathways are complex, however, involving upwards of ten individual steps. In such cases the choice of genes and their organization on a vector becomes of prime importance when considering a recombinant DNA approach to strain development. This invention describes a process for organizing the genes from a biosynthetic pathway on a vector in a manner which maximizes the utility of the recombinant DNA approach to strain optimization and thereby greatly accelerates strain development. The products of this process are termed composite plasmids.

Plasmids containing individual genes or natural operons have been described, for example in L-phenylalanine production FR2486961-A, EP85958-A, GB2053906A, EP77196-A; L-proline production GB2075056-A, EP85958-A; in interferon production, ED126338-A; in tryptophan production, J59125892-A, EP80378A, J57080398-A, US4,371,614, EP124048A. These references do not disclose the composite plasmids described in this document. An amino acid Biosynthesis overview is described in the text "Amino Acids: Biosynthesis and Genetic Regulation", edited by K.M. Herrmann and R.L. Somerville, published by Addison Wesley Pub. Co. (1983).

A review of recombinant DNA procedures can be found in "Molecular Cloning: A Laboratory Manual" by T. Maniatis, E.T. Fritsch and J. Sambrook, published by Cold Spring Harbor Laboratory, NY (1982). U.S. Patent

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4,514,502 describes plasmids containing drug resistance genes and a drive-unit region from a Coryneform glutamic acid-producing bacteria capable of propagating in E.coli or B.subtilis.

5

SUMMARY OF THE INVENTION

As a composition of matter, a composite plasmid comprising:

- 10 (a) a first DNA segment containing a replicon covalently joined to a second DNA segment containing one or more transcriptional units; (b) if only one transcriptional unit, then containing two or more feedback resistant genes coding for two or more enzymes active in
15 the synthesis of an amino acid; (c) if two or more transcriptional units, then a first transcriptional unit containing one or more feedback resistant genes coding for one or more enzymes active in the synthesis of an amino acid and one or more additional transcriptional
20 units containing one or more genes coding for enzymes useful in the production of an amino acid.

Among the amino acids preferred are aromatic amino acids, particularly phenylalanine, tryptophan and tyrosine.

- 25 A preferred embodiment of the invention is a composite plasmid wherein an additional transcriptional unit contains a gene for a catabolic enzyme or a transport protein. A more preferred embodiment is a composite plasmid containing 2 or more feedback resistant
30 genes selected from mutants of the following genes: pheA, aroF, tyrA, pheR, tyrR, tyrB, aroL, aroH, aroG, trpE, aspC, or trp BAD or C. Composite plasmids are capable of replication in procaryotic or eukaryotic
35 cells, given transcription units specific for each cell type.

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One object of the invention is a transcriptional unit containing a catabolic enzyme or a permease useful in amino acid synthesis. A preferred embodiment of the invention is the utilization of an amylase on one of the transcriptional units of the composite plasmid.

Another object of the invention is a method of screening for microorganisms useful in amino acid production wherein a composite plasmid containing a gene coding for a feedback resistant enzyme catalyzing a rate-limiting step in amino acid production is used to transform a cell to be screened, then quantitating the production of the desired amino acid.

The composite plasmids of the invention can be used to screen any Gram-negative organism. Similarly, any Gram negative microorganism can be utilized in amino acid production using the composite plasmids. The composite plasmids exemplified can be used in Gram-negative microorganisms to produce aromatic amino acids, particularly L-phenylalanine and L-tyrosine. Among the Gram negative microorganisms that can be used are the following genera: Neisseria, Veillonella, Brucella, Bordetella, Pasteurella, Haemophilus, Escherichia, Erwinia, Shigella, Salmonella, Proteus, Yersinia, Enterobacter, Serratia, Azotobacter, Rhizobium, Nitrosomonas, Nitrobacter, Thiobacillus, Pseudomonas, Acetobacter, Photobacterium, Zymomonas, Aeromonas, Vibrio, Desulfovibrio, and Spirillum.

Also described is a method for producing a composite plasmid by mutating individual genes to a feedback resistant condition such that the enzymes produced remain active in amino acid synthesis even in the presence of surplus amino acid product, then connecting the mutated genes to a transcriptional unit.

Preferred embodiments of composite plasmids are pME202, pME219, pME214, and pPT112.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 -- Schematic of aromatic amino acid biosynthetic pathway

5 Figure 2 -- Growth curves of HW77/pPT112 on glucose and starch

Figure 3 -- Fermentation of HW77/pPT112 on soluble starch

10 Figure 4 -- Screening strains for phenylalanine production using composite plasmids for L-phenylalanine production (pME202) with zone size measured after 48 hr.

Figure 5 -- Comparison of phenylalanine production by HW77 and HW77/pME202 during fermentation

15

DESCRIPTION OF THE PREFERRED EMBODIMENTS

One object of the invention is to combine on a suitable vector the genes coding for the enzymes that are of greatest importance in influencing the flux of carbon down the biosynthetic pathway of interest. These enzymes are usually the points in the pathway at which regulation principally occurs. In addition to these regulated genes, it is also necessary to include the genes for other enzymes which modulate the regulated steps. These genes usually are in the biosynthetic pathway of interest but they also occur within intermediary metabolism and consequently affect the supply of precursors to the pathway. Other genes may be of more indirect importance. For example, it may be necessary to include: the genes of pathway regulators, either to enhance the flux of interest or to decrease flux through an undesired competing pathway; genes that influence permeability of the cells, to either substrates or products; genes that allow growth of the organism on a more desirable substrate; genes involved

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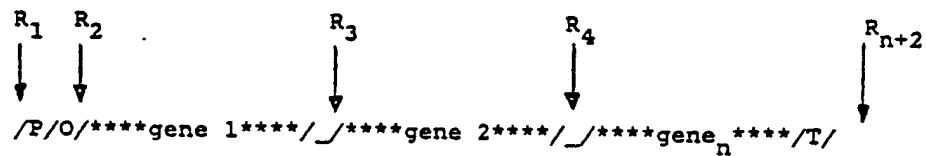
in regulating the cloned genes in such a manner that they can be turned on or off in a manner that facilitates the fermentation; genes that can be used to influence the stability of the plasmid or to select for the plasmid.

The genes of interest for a composite plasmid may optimally be organized as a synthetic or semi-synthetic operon in which all the genes are transcribed from a promoter of the desired regulatory characteristics (see chart 1). Ideally the genes are modified such that they are simply assembled by the use of intervening restriction sites. Use was made of the restriction enzyme BamHI which allows for overlap of both the useful restriction site and a functional ribosome binding site. Finally, it was best to include a transcriptional terminator sequence downstream of the final gene in the operon to limit the extent of the transcript. The whole operon ideally is flanked by restriction sites so that it can be treated as a "cassette", and contain as little redundant DNA as possible to minimize its size. Such an operon was constructed by conventional recombinant DNA techniques, involving initial cloning of the genes followed by the determination of their DNA sequence, directed mutagenesis to introduce the desired restriction sites, and construction of optimal linkers, promoters and terminators by the use of synthetic oligonucleotides. These recombinant DNA techniques are well known to those skilled in the art of genetic engineering.

A transcription unit is defined schematically in Chart 1. It contains assembled components not found together in nature and consists of a regulatory region containing a promoter/operator region, one or more genes mutated to feedback resistance, one or more restriction sites situated between components of the transcription unit and a transcription terminator after the last gene.

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Chart 1 General Structure of an Operon for the Optimum
Organization of Genes



p = promoter

o = operator (may overlap promoter)

Site of desired transcriptional regulation (positive or negative)

(gene) 1 - (gene) n = genes of interest to relevant biosynthetic pathway.

T = Transcriptional terminator.

R_1 - R_n = Restriction sites to facilitate construction and/or manipulation of operon.

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ADVANTAGES OF COMPOSITE PLASMIDS

A composite plasmid is exceedingly versatile and increases the flexibility of the recombinant DNA approach to strain development in the following ways.

1. If the cloned genes for the regulated steps in the pathway have not already been mutated to yield feedback inhibition resistant (FBI^R) derivatives then mutagenesis of the plasmid and screening for producers will quickly yield the desired mutants. This method has the advantage that the recipient background is not subject to mutagenesis and therefore the accumulation of unknown and possibly deleterious mutations that occurs with conventional strain development is avoided.

2. The operon can be simply switched between vectors with different copy numbers to assess the level of expression for optimum productivity.

3. Similarly, the operon can be switched between different vectors to optimize stability. If necessary the operon can be simply reintegrated into the host genetic background.

4. The level of expression can be varied at a given copy number by simple modification to one promoter.

5. The ratio of the different gene products can be finely tuned by altering the efficiency of the ribosome binding sites (RBS). This in turn is greatly facilitated by the presence of restriction sites within the ribosome binding sequences. If necessary a wide disparity in required levels of expression could be accommodated by the use of an attenuator or by splitting the construct into two operons with promoters of different strengths.

6. Since transformation with a plasmid carrying such a construct effectively deregulates a strain

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immediately it is a very rapid method of screening for undefined differences between strains that influence productivity and thus choosing the best background for a producing strain.

5 7. Since such a plasmid can embody all that is known about a pathway it can be used in conjunction with mutagenesis to screen for mutants of a particular strain that offer increased productivity. A strain can be mutagenized followed by transformation and then
10 screened for even more productive strains. This avoids firstly the many rounds of mutagenesis that would be required to deregulate the pathway to the same extent as the plasmid and secondly the potentially deleterious effects of mutagenesis on the cloned genes.

15 8. The operon can be cloned into plasmids with different or extended host ranges to allow for production in different species or genera. The plasmid will be relatively simple to optimize in these backgrounds by virtue of its single promoter and the
20 presence of restriction sites in the RBS sequences.

 9. Additional genes of importance can readily be incorporated as the requirements of the fermentation alter or as understanding of the system evolves.

25 10. Since the complexity of competing transcription/expression is avoided the construction and optimization of plasmids carrying many genes involved with the biosynthesis of a product is made much easier.

DESCRIPTION OF THE EXAMPLES

30

 As examples of the application of this invention we now describe how the biosynthesis of the amino acids phenylalanine and tyrosine can be significantly deregulated and how synthetic operon plasmids can be
35 usefully employed in the development of strains that produce aromatic amino acids, such as L-phenylalanine or L-tyrosine.

- 10 -

The biosynthesis of the aromatic amino acids is an example of a complex pathway that proceeds via a shared common pathway to chorismate where it branches to yield phenylalanine (ten steps), tyrosine (ten steps), tryptophan (twelve steps), the aromatic vitamins and enterochelins, see figure 1.

In E.coli (and other enteric organisms) the crucial step in the regulation of the common pathway is the first reaction catalyzed by the three DAHP synthetase isozymes. The rate of this reaction determines the rate at which carbon is committed to the pathway from the precursors phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). The three isozymes are each inhibited by one of the end products of aromatic amino acid biosynthesis and are coded for by the genes aroF (tyrosine sensitive) aroG (phenylalanine sensitive) and aroH (tryptophan sensitive). In the phenylalanine branch the regulated enzyme is chorismate mutase-prephenate dehydratase, the product of the pheA gene, a bifunctional enzyme in which the prephenate dehydratase activity is very sensitive to feedback inhibition by phenylalanine. Analogously in the tyrosine branch, the tyrA gene product chorismate mutase-prephenate dehydrogenase is sensitive to feedback inhibition by tyrosine.

In addition to feedback inhibition which is the main control operating to regulate carbon flux through the pathway, the levels of the above mentioned enzymes and shikimate kinase (aroL) are all subject to regulation by control at the transcriptional level. The pheA expression is controlled by the phenylalanine repressor, the product of the pheR gene, and by attenuation control. The aroF and tyrA genes together constitute a natural operon and their expression is coordinately regulated by the tyrosine repressor, the product of the tyrR gene. This tyrR also regulates the expression of aroG. Finally, the aroH gene expression

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is regulated by the tryptophan repressor, the product of the trpR gene.

As examples of amino acid production by composite plasmids we constructed plasmids that deregulate either phenylalanine or tyrosine production by suitably engineering the aroF, pheA and tyrA genes from E.coli K12. We used as a source of these genes a 6Kb EcoRI fragment isolated from a gene library by its ability to complement lesions in pheA and aroF G H. This fragment is essentially identical to that described by Zurawski et al. and was cloned into the multicopy vector pAT153 to yield pME65. The pheA and aroF genes were located and sequenced by established methods and deregulated as described in the Examples. Preferred composite plasmids and the host HW77 E.coli were deposited and are listed in the table of deposited plasmids.

Table of Deposited Plasmids

20	HW 77 (Host Cell)	ATCC 13281
	pME 202	ATCC 53136
	pME 214	ATCC 53137
	pME 219	ATCC 53138
	pPT 112	ATCC 53139

25

The American Type Culture Collection is located at 12301 Parktown Drive, Rockville, MD 20852, USA.

30 Example 1 - Preparation of PheA, AroF, AspC and Amylase Genes

1. Preparation of pheA gene

35 The sequence of the pheA gene is described in Chart 2. Insertion of a BamHI-BglII linker at the NcoI

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site was found to render the prephenate dehydratase activity of the enzyme substantially resistant to feedback inhibition by L-phe. In addition, both the chorismate mutase and prephenate dehydratase activities of the enzyme were enhanced. Transcriptional deregulation was effected by replacing the promoter and attenuator sequences with a synthetic promoter based on the natural pheA promoter but lacking the dyad symmetry overlapping the pribnow box sequence (-10). This replacement was made between the EcoRI site before the gene and a HaeII site lying in the N-terminus of the natural pheA gene. Finally, a BamHI site was introduced 3' to the pheA gene by directed mutagenesis. The resulting optimized pheA sequence is illustrated in Chart 3.

2. Preparation of aroF gene

The sequence of the aroF gene is described in Chart 4. A feedback inhibition resistant derivative was isolated in the following way: The aroF gene was first subcloned on a HindIII-BamHI fragment into the intermediate copy number vector pLG338. This was transformed into a strain (HW295) lacking all three DAHP synthetase isozymes. This resultant strain was then able to grow on minimal medium in the absence of exogenous aromatic amino acids and shikimate by virtue of the wild type aroF gene product carried on the plasmid. In the presence of 100mcg tyrosine ml^{-1} , however, the aroF gene product is inhibited and consequently the strain is unable to synthesize sufficient Phe and Trp for growth. Mutants capable of growth on minimal media were isolated, pooled and retransform HW295 and the resulting transformants were screened for their ability to grow on minimal medium supplemented with tyrosine. Transformants capable of growth on this medium were assumed to have received a mutant plasmid and were

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EcoRI
 GAATTCACCAAGACGGGAGACAAGAGCTAAAATTTATGACATGAACATTACCAGCAA 68

 CAAATGGAAATTACTCGGCCATCGCCAACATGTGCGAGACGCTCTGCCAAACTGGAA 128
 AAATGGCAACACATCTGATTAATCCACATATCATCTGTCCAAAGAGCCCAAGGGTTT 188
 GTTGTGACGCGCAATCAATACACCTAACGGGCTTCTGGTTCAGTGGTAAACATGAA 248
 GATATGTACACCGCAATTAACGAATTGATCAACAAGCTGGAACGGCAGCTCAATAAACTG 308
 CAGCAAAAGGGGAAGAGCTGTCGCAACATCGGTGAAGACGCCAAGCTTGGTGAAG 368
 AAGTTGAAGAAGAGTAGTCCCTTATATGAGTGTATGCGCAACGCGCTTGGGGCGCTT 428
 TTTTGTGACAGCGTGAACAGTACGGGTACTGTACTAAAGTCACTTAAGGAAACAAAC 488
 N K H I P F F P A P F F T P P
 ATGAAACACATACGCTTTTCTTCGCAATCTTTTACCTCCCTGAATGGGAGCGTT 548
 TCGTGTGTGAACAGAAATGGAAGACGAACAATAAGGCCTCCCAATCGGGGGCGCTT 608
 M T S E N P L L A L R
 TTTATTGATAACAAAAGGCAACACTATGACATGGGAAAACCGTTACTGGCGCTGCGAG 668
 E R I S A L D E K L L A L L A E R R E L
 AGAAAAACAGCGCGCTGGATGAAAAATTATTAGCGTTACTGGCAGAAAGGGCGGAACCTGG 728
 A V E V G K A K L L S H R P V R D I D R
 CGGTGAGGTGGGAAAGCCAACTGCTCTGCGATCGCCGCTAAGTGTATATGATGCTG 788
 E R D L L E R L I T L G K A H H L D A H
 AAGCGATTTCGTGGAAGATTAAATTACGCTCGGTAAAGCGCACTCTGGAGCGCCATT 848
 Y I T R L F Q L I I E D S V L T Q Q A L
 ACATTACTGCGCTGTCAGCTCATCTGAAGATTCTGATTAACTCAGCAGGCTTTGC 908
 L Q Q H L N K I N P H S A R I A P L G P
 TCCAACAACATCTCAATAAAATTAATCGCACTCAGCAAGCATCGCTTTCTCGGCCCA 968
 K G S Y S H L A A R Q Y A A R H F E Q F
 AAGGTTCTTATCCCATCTGTGGGGCGCCAGTATGCTGCCGTCACCTTGAGCAATTCA 1028
 I E S G C A K P A D I F N Q V E T G Q A
 TTGAAGTGGCTGCGCAAATTTCCEATATTTTAAATCAGGTGGAACCGGCCAGGCCG 1088
 D Y A V V P I E N T S S G A I N D V Y D
 ACTATGCGGTGACGATTTGAAAATACAGCTCGGTGCCATAAAGACGTTTACGATC 1148
 L L Q H T S L S I V G E M T L T I D H C
 TGCTGCAACATACCAGCTTGTGATTTGTCGAGATGACGTTAACTATCGACCATTTGT 1208
 L L V S G T T D L S T I N T V Y S H P Q
 TGTGGTCTCGGCACTACTGATTTATCCACCATCAATACGGCTACAGCCATCGCAGC 1268
 P P Q Q C S K P L N R Y P H W K I E Y T
 CATTCAGCAATGCAAGCAATTCCTTAATCGTTATCGCACTGGAAGATTGAATATACG 1328
 E S T S A A M E K V A O A K S P H V A A
 AAAGTACGTCGTGCGCAATGGAAGGTTGCAAGGCAAAATCACCGCATGTTGTGCGT 1388
 L G S E A G G T L Y G L Q V L E R I E A
 TGGGAAGCGAAGCTGGGGCACTTTGTACGTTTGAGGTACTGGAGGCTATTGAAGCAA 1448
 N O R O N P T R P V V L A R K A I N V S
 ATCAGCGACAAAACCTCACCGATTTGTGGTGTGGGCGGTAAAGCCATTAAAGTGTCTG 1508
 D Q V P A K T T L L M A T G Q Q A G A L
 ATCAGGTTCCGCGCAAAACAGTTGTTAATGGGACCGGGCAACAAGCGGTGCGCTGG 1568
 V E A L L V L R N H N L I M T R L E S R
 TTGAAGCGTTGCTGTACTGCGCAACCAATCTGATTATGACCGCTCTGGAATCAAGCC 1628

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P I E G N P W E E M P Y L D I Q A M L E
 CGATTCAAGGTAATCCATGGGAAGAGATGTTCTATCTGGATATTCAGGCCAATCTTGAAT 1688
 333333
 S A E M Q K A L K E L G E I T R S M K V
 CAGGGGAATGC AAAAGCATTGAAAGAGTTAGGGGAATCACCGTTCAATGAAGGTAT 1748
 L G C Y P S E N V V P V D P T ***
 TGGGCTGTTACCCAGTGAGAAAGTAGTGCCGTGTCATCCAACTGATGAAAAGGTGCCG 1808
 GATGATGTAATCATCGGCCACTGGATTATTACTGGGATTTGTCATTGCGCTGACGCAAT 1868
 AACACGCGGCTTTCACTCTGAAAAGCGTGTGGTAATGCGGAACGAGTGCTCCACCTTG 1928
 CGGAAACTGTCAATAAAGCGCTGCTTATGCGCCGTGCTCGAGCACTCAATGCGCTGCGG 1988
 AAAGCTTATAGTAAGCTTTGATTAAAGCCGAGATTACGCTCTGAGACATAATGATGTGG 2048
 GCATAAGCTGCGGATCC 2058

BamBI

Chart 2 Sequence of the EcoRI-BamBI Fragment Encompassing
 The *phsA* Gene.

KEY

The BaeII site used in the promoter swap 333333
 The NcoI site used for the BglII linker mutagenesis 333333

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EcoRI      -35      PROMOTER      -18      SD
GAATTCCTTTTGTGTGACAGCGTGAACAGTACGGGTATAACTAAAGTCACAAAAG 68
-----
          M T S E N P L L A L R E K I S A L
GCAACACTATGACATGGGAAAACCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGG 128
          111111
D E K L L A L L A E R R E L A V E V G K
ATGAAAATATTAGCGTTACTGGCAGAACGCGCGAACTGCGCGTGGAGCTGGGAAAAG 168
A K L L S H R P V R D I D R E R D L L E
CCAACTGCTCTCGCATCGCCGCTAGCTGATATTGATCGTGAACGCGATTGCTGGAAA 248
R L I T L G K A H H L D A N Y I T R L F
GATTAATTAGCTCGGTAAGCGCACATCTGGACGCCATTACATTACTCGCGTGTTC 388
Q L I I E D S V L T Q Q A L L Q Q H L H
AGCTCATCTGAAGATTCCTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATA 368
K I N P H S A R I A P L G P K G S Y S H
AAATTAATCGCACTCAGCAGCATCGCTTTCTCGGCCCAAGGTTCTTATTCCTATC 428
L A A R Q Y A A R H P E Q P I E S G C A
TTGGGCGCGCCAGTATGCTGCCGTCACTTTGAGCAATTCAATTGAAGTGGCTGGCCA 488
K P A D I P H Q V E T G Q A D Y A V V P
AATTTCGATATTTTAAATCAGGTGGAACCGGCCAGGCGACTATGCGTCTGTACCA 548
I E N T S S G A I N D V Y D L L Q H T S
TTGAAAATACAGCTCGCGTGCATAAAGAGCTTTACGATCTGCTGCAACATACAGCT 688
L S I V G E N T L T I D H C L L V S G T
TGTCGATGTTGGCGAGATGACGTTAACTATCGACCATGTTGTTGTTGCTCTCGGCACTA 668
T D L S T I N T V Y S H P Q P P Q Q C S
CTGATTTATCCACCATCAATACGGTCTACAGCCATCGCAGCCATTCAGCAATGAGCA 728
K F L N R Y P H W K I E Y T E S T S A A
AATTCCTTAATCGTTATCGCACTGGAAGATTGAATATACGAAAGTACGTCGCGGCA 788
H E R V A Q A K S P H V A A L G S E A G
TGGAAAAGGTTGCAAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCG 848
G T L Y G L O V L E R I E A N O R O N P
GCACITGTAGCGTTTGAGGTACTGGAGGTATTGAAGCAATCAGCGACAAAATTCA 988
T R F V V L A R K A I N V S D Q V P A K
CCCGATTGTGGTGTGGCGCGTAAAGCCATTAACGTGTCGATCAGGTTTCGGCGAAAA 968
T T L L M A T G Q Q A G A L V E A L L V
CCAGTTGTTAATGGCGACCGGCAACAGCGGTGCGCTGTTGAAGCGTTGCTGGTAC 1828
L R N H N L I H T R L E S R P I H G N P
TGCGCAACCAATCTGATTATGACCGTCTGGAATCAGCGCGATTCAAGGTAATCCAT 1888
W R S P W E E N P Y L D I Q A N L E S A
GGAGATCTCCATGGGAAGAGATGTTCTATCTGATATTCAGGCCAATCTTCAATCAGCGG 1148
E H Q K A L I K E L G E I T R S H K V L G
AAATGCAAAAAGCATGGAAGAGTTAGGGGAAATCACCGTTCAATGAAGGTATTGGGCT 1288
C Y P S E N V V P V D P T *****
GTTACCCAAGTGAGAAGTAGTGCCTGTTGATCCAACCTGATGAAAAGGATCC 1253
          BamHI

```

Chart 3 Optimised *phsA* Sequence.

The sequence of the EcoRI-BamHI fragment carrying the *phsA* gene from pME198 is presented.

KEY

The HaeII site used in the promoter swap
 The 12 bp insertion conferring FBI-R
 The Shine & Dalgarno sequence in the RBS.
 The -18 and -35 regions of the promoter

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          111111
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          6666
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518DIII
AAGCTTTTACCGGAAAGTCCTCGGGCATAGTAAATCGGTCTGCGCAAGTTTCTTTTGT 60
CCCTCGGCATATCTTTAAGGTATAGACCGGATCGCGCTGGAATCGCTAAATGCATCG 120
TCATCTTTTATGGCGCTGCCGATATCGCATCTTCCATACGACATCATACAGTGGTG 180
CTGGTCAGGTGGCGGTGAGTATAAGGATCAATCTGACATCGCTAACCGTAAGCC 240
AACAAGGGGGGATAACTTTTTCATCTTCTTTCTCTTTTCAAAGCATAGCGATT 300
GTTTCAAAGGGAGTGTAAATTTATCTATACAGAGGTAGGGTTGAAGCGGACTAAAT 360
TGCCCTGTAAATAAAATGTACGAAATATGGATTGAATACTTTACTTTATGTGTTATCG 420
TTAGCTCATCTCTCGCTGAGGATCAACTATCGCAAAGAGCAATAACAGGATCGCATCAT 480
G. K D A L N N V H I T D E Q V L M T F E
GCAAAAGACCGCGCTGAATAACGTACATATTACCGAAGAGGTTTAAATGACTCGCGA 540
Q L K A A P P L S L O Q E A Q I A D S R
ACAACGTAAGGGCGCTTTTCCATTGAGCTGCAACAAGAGCCGAGATTGCTGACTCGCG 600
K S I S D I I A G R D P R L L V V C G P
TAAAGCATTTTCAGATATTATCGCGGGCGGATCTCTGCTGCTGGTAGTATGTGGTCC 660
C S I H D F E T A L E Y A R R F K A L A
TTGTTCCATTGATCGGAAACTGCTCTGGAATATGCTGCTGATTTAAAGCCCTGTC 720
A E V S D S L Y L V M R V Y F E K P R T
CGCAGAGGTGAGCGATGCGCTCTATCTGTAATGCGCTCTATTGAAAACCCGCTAC 780
T V G W K G L I N D P E H M D G S P D V E
CACTGTGCGCTGGAAGGGTTAATTAAAGATCCCATATGGATGGCTCTTTGATGTAGA 840
A G L Q I A R K L L L E L V H M G L P L
AGCGGGCTGCGAGATCGCGCTAAATGCTGCTGAGCTGGTAATATGGGACTGCCACT 900
A T E A L D P H S P Q Y L G D L F S W S
GGTACGGAAGCGTTGATCGGAATAGCCGCAATACCTGGGCGATCTGTTTATGCTGGTC 960
A I G A R T T E S O T H R E M A S G L S
AGCAATTGGTGCTGTACAAGGGAATCGCAAACTCACCGTGAATGGCTCGGGCTTTC 1020
K P V G F K N G T D G S L A T A I N A M
CATGCGGTGGTTTAAAAACGGCAAGCGGAGTCTGGCAACAGCAATTAAGCTAT 1080
R A A A Q P H R P V G I N Q A G Q V A L
GCGCGCGCGCGCGCGCGCTTTTGTGGCATTAAACAGGCGAGGGCAGGTTGCGTT 1140
L O T Q G N P D G H V I L R G G K A P N
GCTACAAACTCAGGGGAATCGGAGCGGCAATGATCTGCGCGGTGTAAGCGCGAA 1200
Y S P A D V A Q C E K E M E Q A G L R P
CTATAGCCCTGGGATGTTGCGCAATGTAAGAGATGSAACAGGGGACTGCGCGC 1260
S L M V D C S E G H S H E D Y R R Q P A
GTCTCTGATGTTGATTGCAAGCGGTAATTCGAATAAGATTATCGCGTCAAGCTGC 1320
V A E S V V A Q I K D G H R S I I G L M
GGTGGCAGATCGGTGCTCAATCAAGATGGCAATCGCTCAATTATGCTGCTGAT 1380

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I E S M I E E G N O S S E O P R S E N E
 GATCGAAGTAATATCCAGAGGGCAATCAGTCTTCGAGCAACCGGCAGTGAATGAA 1448
 Y G V S V T D A C I S W E N T D A L L R
 ATACGGTGATCCGTAAACGATGCCCTGCATTAGCTGGGAAATGACGATGCCTTGTCTGCG 1588
 E I N Q D L N G Q L T A R V A ***
 TGAATTCATCAGGATCTGAACGGCCAGCTGACGGCTCGCTGCTTAAGAGGTTTATTA 1568
 TGGTTCCTGAATTGACCGCATTACCGGATCAAATTGATGAAGTCGATAAAGCGCTGCTGA 1628
 ATTTATTAGCGAAGCGTCTGGAACCTGGTTGCTGAAGTGGCGAGGTGAAAAGCGCTTTG 1688
 GACTGCCATTTATGTTTCGGCAGCGCGAGGCATCTATGTTGGCCTCGCTGCTGACAG 1748
 CGGGAAGCTCTGGGTGTACCGCCAGATCT 1769

 Bgl II

Chart 4 Sequence of the HindIII-BglII Fragment Encompassing
 the *arp* Gene.

KEY

Sequence altered to a BamHI site in EES
 Codon altered in FBI-R mutant (CAAasn-CGAarg)
 PvuII site used in terminator swap underlined

 1
 1111111

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assayed for DAHP synthetase activity. One mutant that carried a substantially feedback inhibition resistant aroF gene was kept for further analysis. Restriction fragment switching experiments revealed that the feedback lesion was located on a BstXI to PvuII fragment. Subsequent DNA sequencing revealed a single base pair alteration that changed glutamine 152 to an arginine residue.

The promoter and tyrR binding site were removed by introducing a BamHI site into the aroF ribosome binding site. Sequences downstream of the aroF gene were removed by replacing a PvuII-SalI fragment with a synthetic terminator also carried on a PvuII-SalI fragment. The sequence of the optimized aroF is illustrated in Chart 5.

In addition to pheA, aroF and tyrA genes, the aspC gene from E.coli K12 and the alpha amylase gene from B.licheniformis were prepared for use in composite plasmids.

3. Preparation of aspC gene

The cloning of the aspC gene has been described in European Patent Application 84100521.8 (Pub. No. 116860). For the purposes of the present invention it was necessary to sequence the gene and introduce convenient restriction sites. The DNA sequence is presented in Chart 6. A BamHI site was introduced into the ribosome binding site by directed mutagenesis. A BglII site was introduced downstream of the gene by making use of a convenient StuI site to insert a BglII linker. This enabled the aspC gene to be isolated on a convenient BamHI to BglII fragment. The optimized aspC gene is shown with ribosome binding site in Chart 7.

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4. Preparation of Amylase gene

The cloning of the B.licheniformis gene is described in European Patent Application 84308868.3.

- 5 The nucleic acid sequence for the amylase gene is shown in Chart 8. Into the ribosome binding region we introduced a BamHI site by directed mutagenesis. A second BamHI site was introduced downstream of the gene by inserting BamHI linkers into a convenient HindIII
10 site. Details of these modifications are described in Chart 9.

Examples of Composite plasmids and their applications

15 Example 2

Constructs in which the genes are transcriptionally deregulated by virtue of their high copy number.

- 20 The plasmid pME157 carries a FBI^R pheA gene transcribed from its wild type promoter cloned into the high copy number vector pAT153. The aroF gene along with a portion of the tyrA gene on a HindIII-BglII fragment was first modified by the addition of a BglII
25 linker to the HindIII end and then cloned into the BamHI site of pME157. Two classes of plasmid resulted from this procedure. In the first (pME171) the pheA and aroF gene are transcribed colinearly. The second class (pME170) carries the two genes transcribed conver-
30 gently. pME171 was then modified to encode a FBI^R aroF product by switching a BstXI fragment with the FBI^R aroF plasmid pMX13 to yield the deregulated plasmid pST35. The effect of these plasmids on the levels of DAHP syn-
35 thetase and prephenate dehydratase are summarized in Table 1.

- 20 -

```

BamHI      H Q R D A L N H V E I T D E O V
GGATCCCCATCATGCAAAAAGACGCGCTGAATAAGGTACATATTACCGACGAACAGGTTT 68
-----
L N T P E Q L K A A P P L S L O Q E A Q
TAATGACTCCGGAACACTGAAGGCCGCTTTTCATTGAGCCTGCAACAAGAACCCGGA 128
I A D S R E S I S D I I A G R D P R L L
TTGCTGACTCGGTAAGAATTTTCAGATATTATCGCGGGCGGATCCTCGTCTGCTGG 188
V V C G P C S I E D P E T A L E Y A R R
TAGTATGTGGTCTGTGTTCCATTCAATGATCGGAAACTGCTCTGGAATATGCTCGTAT 248
P K A L A A E V S D S L Y L V H R V Y P
TTAAGGCCCTGCGCGAGAGGTGAGCGATAGCCTCTATCTGCTAATGCGGCTCTATTG 308
E R P R T T V G W K G L I N D P H N D G
AAAAACCCGTACCACTGTGGCTGGAAGGTTAATTAAGATCCCATATGCGATGGCT 368
S P D V E A G L Q I A R K L L L E L V N
CTTTGATGTAGAAGCGGGCTG CAGATCGCGGTAAATGCTGCTGAGCTGGTGAATA 428
H G L P L A T E A L D P H S P R Y L G D
TGGGACTGCCACTGGGAGCGGAAGCGTTAGATCCGAATAGCCCGGATACCTGGGCGATC 488
L P S W S A I G A R T T E S O T H R E N
TGTTTAGCTGCTCAGCAATTGGTGTCTGTACAAGGAATGCAAACTCAGCGTGAATGG 548
A S G L S H P V G P E H G T D G S L A T
CCTCGGGCTTTCCATGCGGGTTGTTTTAAAAAGCGCACCGAGCGAGTCTGGCAACAG 608
A I N A M R A A A Q P H R P V G I N Q A
CAATTAAGCTATGCGCGCGCGCCGAGCGCGACGTTTTTGTGCAATTAACGAGGCG 668
G Q V A L L Q T O G H P D G H V I L R G
GGCAGGTTGCTTGTCTACAACTCAGGGGAATCGGAGCGCATGTGATCCTGCGCGTG 728
G K A P H Y S P A D V A Q C E K E H E Q
GTAAAGCGCGAACTATAGCCCTGCGGATGTTGCGCAATGTGAAAAGAGATGGAACAG 788
A G L R P S L H V D C S H G H S H K D Y
CGGACTGCGCCGTCTCTGATGTTAGATTGCGGCGGTAATTCATAAAGATTATC 848
R R Q P A V A E S V V A Q I K D G H R S
GCGGTGAGCCTGCGGTGGCAGAAATCGTGTGCTCAATCAAGATGCAATCGCTCAA 908
I I G L H I E S H I E G H Q S S E Q P
TTATGCTGATGATGCAAAAGTAATATCCAGGAGGCAATCAGTCTTCCGAGCAACGC 968
R S E H E Y G V S V T D A C I S W E M T
GCAGTGAATGAATAAGGTGATCTGTAACGATGCTGCTAGCTGGGAAATGACCG 1028
D A L L R E I H O D L H G Q L T A R V A
ATGCGTGTGCTGCGTGAATTCATCAGGATCTGAACGGGCGAGCTGACGGCTCGCTG 1088
*****BamHI      Sali
AATGAGGATCCCGGGGATTCTACGCCCGGTTTTTATGTCGAC 1125
-----
          $$$$$$          $$$$$$
          SmaI          SmaI
          -----

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Chart 5 Optimised *arp* Clone.

KEY

Site of FBI-R mutation
 SmaI sites in terminator
 Dyad symmetry in terminator
 PvuII site used in terminator swap underlined

*10
 \$\$\$\$\$\$

 \$\$\$\$\$\$

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TTCTAATAGCACACTCTTTGTTAAATGCCGAAAAAACAGGACTTTGGTCTCTTTTTT 60
TATACCTTCCAGAGCAATCTCACGTCTTGCAAAAACAGCCTGCGTTTTTCATCAGTAATAG 120
TTGGAATTTTGTAAATCTCCCGTTACCTGATAGCGGACTTCCCTTCTGTAACCATAATG 180
      N F E N I T A A P A D F I L G L A
GAACCTGCTCATGTTTGAGAACATTACCGCCGCTCTGCGGACCCGATTCGCGCCCTGGC 240
      D L F R A D E R P G R I N L G I G V Y R
CGATCTGTTTCTGCGGATGAACGTCCCGCAAAATTAACTCGGGATTGGTGCTATAA 300
      D E T G K T P V L T S V K K A E Q Y L L
AGATGAGACTGGCAAAACCCGCTACTGACGAGCGTGAAAAAGGCTGAACAGTATCTGCT 360
      E N E T T E N Y L G I D G I F E F G R C
CGAAAAATGAACCAACCAAAATTACCTCGGCATTGACGGCATCCCTGAATTGCTGCTG 420
      T O E L L F G K G S A L I N D E R A R T
CACTCAGGAAGTCTGTTTGTAAAGGTAGCGCCCTGATCAATGACAAACGTGCTCGCAC 480
      A Q T P G G T G A L R V A A D P L A E N
GGCACAGACTCCGGGGGGCACTGGCGCACTACGCGTGGCTGCGGATTTCCTGGCAAAAA 540
      T S V R R V W V S N P S W P N E K S V F
TACCAGCGTTAAGCGTGTTGGGTGAGCAACCAAGCTGGCGCAACCAAGAGCGCTCTT 600
      M S A G L E V R E Y A Y Y D A E N N T L
TAACTCTGAGGTCTGGAAAGTTCTGTAATACGCTTATTATGATGCGGAAATCAGACTCT 660
      D F D A L I N S L N E A Q A G D V V L F
TGACTTCGATGCACTGATTAACAGCCTGAATGAAGCTCAGGCTGGCGCAGTGTCTGTT 720
      H G C C H N P T G I D P T L E Q W Q T L
CCATGGCTGCTGCCATAACCCCAACCGGTATCGACCCCTACGCTGGAACAATGGCAACACT 780
      A Q L S V E K G W L P L P D P A Y Q G F
GGCACAAGTCTCCGTTGAGAAAGGCTGCTTACCGCTGTTTGACTTCGCTTACGAGGTTT 840
      A R G L E E D A E G L R A P A A N E K E
TGCCCGTGGTCTGGAAGAAGATGCTGAAGGACTGCGCGCTTTCGCGGCTATGCATAAAGA 900
      L I V A S S Y S K N F G L Y N E R V G A
GCTGATTGTTGCCAGTTCTTACTCTAAAAACTTTGGCCTGTACACGAGCGTGTGGCGC 960
      C T L V A A D S E T V D R A P S O N K A
TTGTACTCTGGTTGCTGCCGACAGTGAAACGTTGATCGCGCATTGAGCCAAATGAAGC 1020
      A I R A N Y S N P P A N G A S V V A T I
GGCGATTGCGCTAACTACTCTAACCCACGACACGCGCTTCTGTTGTTGCCACCAT 1080
      L S N D A L R A I W E Q E L T D N R O R
CTTGAGCAACGATCGGTTACGTCGATTGCGGAACAAGAGCTGACTGATATGCGCCAGCG 1140
      I Q R N R O L F V N T L Q E R G A N R D
TATTCAGCGTATGCGTCAGTTGTTCTCAATACGCTGCAGGAAAAAGCGCAAAACCGCGA 1200
      F S F I I K Q N G H P S F S G L T R E Q
CTTCAGCTTTATCATCAAAACAGACGGCATGTTCTCTTTCAGTGGCTGACAAAAGAACA 1260
      V L R L N E E F G V Y A V A S G R V N V
AGTGCTGCGTCTGCGGAAGAGTTTGGCGTATATGCGGTTGCTTCTGCTCGCGTAAATGT 1320
      A G N T P D N H A P L C E A I V A V L ...
GGCGGGGATGACACCAGATAACATGGCTCCGCTGTGCGAAGCGATTGTGGCAGTCTGTA 1380
      AGCATTAAAAACAATGAAGCCCGCTGAAAAGCGGGCTGAGACTGATGACAAACGCAACAT 1440
TGCTGATGCGCTACGCTTATCAGGCT 1468

```

Chart 6 Natural *aspC* Gene Sequence.

KEY

Site of *Bam*HI site introduced in RBS

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Table 1 DAHP Synthetase and Prephenate Dehydratase Activities of HW77 Carrying Various Phe Plasmids.

5	PLASMID	PREPHENATE	DAHP
		DEHYDRATASE	SYNTHASE
10	HW77 alone	ND	ND
	pME214	19	65
	pME208	36	49
	pMH19	141	324
	pME202	705	1145
15	pME219	491	694
	pME170	290	4505
	pME171	372	3570
	pST35	546	7433
	pME237	240	3146
20	pME238	209	3420
	pAT153	3	34

HW77 was transformed with the plasmids indicated. Each strain was grown to OD₆₀₀ 1.0 in minimal medium supplemented with 0.5% casamino acids. Cell extracts were then prepared and used to assay the activities of prephenate dehydratase and DAHP synthase. Prephenate dehydratase activity was measured in the presence of L-phenylalanine. DAHP synthase activity was measured in the absence of feedback inhibitors and so represents the total DAHP synthase activity. Enzyme activities are expressed in mU min⁻¹ mg protein⁻¹.

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The three plasmids pME171, pME170 and pST35 were introduced into the tyrA organism HW77. Cross-feeding experiments on these strains (Table 2) showed that the plasmids pME170 and pME171 caused a significant production of L-phenylalanine. HW77 carrying pST35, however, grew very badly on minimal medium and as a consequence showed very little cross-feeding. It appears that a high level of FBI^R aroF is deleterious to the cell and that lower levels of the enzyme are required for optimum L-phenylalanine production under these conditions.

Example 3

Composite plasmids which include a transcriptionally deregulated pheA gene.

The plasmid pJW2 carries the FBI^R pheA gene transcribed from the synthetic promoter. Plasmid pME227 carries a FBI^R aroF gene transcribed from its own promoter and ending with the synthetic terminator sequence described above. Both plasmids are based on the multicopy plasmid pAT153. An EcoRI fragment carrying pheA was constructed by the addition of EcoRI linkers to an EcoRI-BamHI fragment of pJW2 carrying pheA. This fragment was then cloned into the EcoRI site of pME227. The resulting plasmids carrying pheA transcribed in both orientations relative to aroF were designated pME238 (divergent) and pME237 (colinear). The DAHP synthetase and prephenate dehydratase activities in strains carrying these plasmids are given in Table 2.

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Table 2 Cross-Feeding Experiments on HW77 Carrying
Cloned Genes Pertaining to Phenylalanine Production
(Phe Plasmids).

5 HW77 was transformed with all the plasmids
described in the examples section to give the strains
listed below. The relative extent of cross-feeding
after 24 hr and 48 hr is indicated.

10	PLASMID	CROSS-FEEDING OBSERVED	
		24 hr	48 hr
	HW77 alone	(+)	+
	pME214	++	++++
15	PME208	++	++++
	pMH19	+++	+++++
	pME202	+++	+++++
	pME219	++	++++
	pME170	++	++++
20	pME171	++	++++
	pST35	NG	(+)*
	pME237	NG	(+)*
	pME238	NG	(+)*
	pPT112	++	+++
25	pAT153	(+)	+

* Some cross-feeding observed but from what were
obviously a few mutant colonies that had managed to
grow.
30 NG = no growth

Both plasmids when introduced into HW77 (ATCC
13281) prevented the strain from growing on minimal
35 media. Again this is probably due to the excessive
expression of FBI^R aroF. As a result of this neither

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888MI      M F E N I T A A P A D P I L G L
GGATCCTGTCATGTTTGAGAACATTACCGCCGCTCTGCGGACCGGATTCTGGGCGCTGG 60
.....
A D L P R A D E R P G K I M L G I G V Y
CCGATCTGTTTCGTCGCGATGAACGTCCCGGCAAAATTAACCTCGGGATTGGTGCTATA 120
K D E T G E T F V L T S V K E A E O Y L
AAGATGAGACGGGCAAAACCCCGTACTGACCAGCGTGAAAAAGGCTGAACAGTATCTGC 180
L E N E T T E N Y L G I D G I F E F G R
TCGAAATGAACACCAAAATTAACCTCGGCATTGACGGCATCCCTGAAATTTGGTCGCT 240
C T Q E L L F G K G S A L I N D E R A R
GCACTCAGGAACGTGCTGTTTGGTAAAGGTAGCGCCCTGATCAATGACAAACGTGCTCGCA 300
T A Q T P G G T G A L R V A A D P L A K
CGGCACAGACTCCGGGGGCACTGGCCCACTACGCGTGGCTGCGGATTCTCGGCAAAA 360
N T S V K R V W V S H P S W P N H K S V
ATAACAGCGTTAAGCGTGCTGGGTGAGCAACCCAAGCTGGCGGAACCAAGAGCGTCT 420
F N S A G L E V R E Y A Y Y D A E N N T
TTAAGCTGTCAGGTTCTGGAAGTTCTGGAATACGCTTATTATGATCGCGGAAATCAACTC 480
L D P D A L I N S L N E A Q A G D V V L
TTGACTTCGATGCACTGATTAACAGCGCTGAATGAAGCTCAGGCTGGCGACGTAGTCTGT 540
F H G C C H N F T G I D P T L E Q W Q T
TCCATGGCTGCTGCCAACAACCCGCTATCGACCTACGCTGGAAACATGGCAACAC 600
L A Q L S V E K G W L P L P D F A Y Q G
TGGCAAACTCTCGGTTGAGAAAGGCTGGTTACCGCTGTTTGACTTCGCTTACCAGGTT 660
F A R G L E E D A E G L R A F A A N N K
TTGCCCGTGCTCTGGAAGAAGATGCTGAAGGACTGCGCGCTTTGCGGCTATGCAZAAAG 720
E L I V A S S Y S E N F G L Y N E R V G
AGCTGATTGTTGCCAGTTCCTACTCTAAAACTTTGGCCTGTACAACGAGCGTGTGGCG 780
A C T L V A A D S E T V D R A F S Q H K
CTTGACTCTGGTTGCTGCGGACAGTGAAACCGTTGATCGCGCATTGAGCCAAATGAAAG 840
A A I R A N Y S N P P A H G A S V V A T
CGGCGATTGCGGCTAACTACTCTAACCCACAGCACACGGCGCTTCTGTTGTTGCCACCA 900
I L S N D A L R A I W E Q E L T D N R O
TCCTGAGCAACGATGCGTTACGTGCGGATTGGGAACAGAGCTGACTGATATGCGCCAGC 960
R I Q R N R O L F V N T L Q E R G A N R
GTATTGAGCGTATGCGTCAGTTGTTGCTCAATACGCTGCGGAAAAAGGCGCAACCGCG 1020
D F S F I I K Q H G N F S F S G L T K E
ACTTCAGCTTTATCATCAACAGAACGGCATGTTCTCTTCAGTGGCCTGACAAAGAAC 1080
Q V L R L N E E F G V Y A V A S G R V N
AAGTGCTGCGTCTGCGCGAAGAGTTTGGCGTATATGCGGTTGCTTCTGGTCCGCTAAATG 1140
V A G N T P D N N A P L C E A I V A V L
TGGCGGGGATGACACCAGATAACATGGCTCCGCTGTGCGAAGCGATTGTGGCAGTCTGT 1200
..
AAGCATTAAAAACAATGAAGCCCGCTGAAAAGCGGGCTGAGACTGATGACAAACGCAACA 1260
89111
TTGCGCTGATGCGCTACGCTTATCAGGAGATCT 1293
.....

```

Chart 7 Optimised aspC Sequence.

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EcoRI
 GAATTCAGATTTTCTGAACAGGATTTCAAGCATTGCCATCAGGAATTGAAAAATCGGAA 60

 TCCGCTCGCTGACATGGCTTTATTAAAGAAGGGACGAGCCCTCAGGAAGAACATTTTCAG 120
 CCGCTTTGGCCTTCTTGAACAGGAGTGGAAATTTTATTTTGAGAAAAACAAACCGGTCT 180
 TGAACAGTCTGTAAAAATAAAAAAGAATGGGTAAAGATATTTTAAAGACCGATATCA 240
 GGATATGAAAAAGAAATCGTCTTCAGGCCAAACCTGATCAGGAGCCTGTTCCGCTTCGGAA 300
 GCAAGCGAAAAATTAATCCCGATGAAAAAGTGATTGCCCTCAGATTTGATCAGCGTCCGAA 360
 TCCCGGTACAACGAATAAAAAATATTAAACGCTTTACAGAAGCATGAAGGGCATGCGACCTT 420
 CTTTGTGCTTGGAAAGCAGAGCCCAATATTATCCCGAAACGATAAAACGGATGCTGAAGGA 480
 AGGAAACGAAGTCGGCAACCATTCCTGGGACCATCCGTATTGACAAGGCTGTCAAACGA 540
 AAAAGCGTATCAGGAGATTACGACACGCAAGAAATGATCGAAAAAATCAGCGGACACCT 600
 GCCTGTACACTTGGCTCTCCATACGGCGGGATCAATGATTCGCTCCGCTCGCTTTCCAA 660
 TCTGAAGGTTTCATTGTGGGATGTTGATCCGGAAGATTGGAAGTACAAAAATAAGCAAAA 720
 GATTGTCAATCATGTCATGAGCCATGCGGGAGACGGAAAAATCGTCTTAATGCACGATAT 780
 TTATGCAACGTCGCGAGATGCTGCTGAAGAGATTATTAAGAGCTGAAGCAAAAGGCTA 840
 TCAATTGGTAACTGTATCTCAGCTTGAAGAGTGGAAGAGCAGAGAGGCTATTGAATAAA 900
 TGAGTAGAAGCGCCATATCGCGGCTTTCTTTTGGAGAAAATATAGGGAATGGTAC 960
 TTGTTAAAAATTCGGAATATTATACAAATCATATGTTTCACATTGAAGGGGAGGAGA 1020
 N K Q Q K R L Y A R L L T L L P A L I
 ATCATGAACACAAAAACGGCTTTACGCCGATTCGCTGACGCTGTTATTTGGCTCATC 1080
 F L L P H S A A A A A H L N G T L H O Y
 TTCTTGCTGCTCAATCTGACGAGCGGGCGCAATCTTAATGGGACGCTGATGCAGTAT 1140
 F E W Y H P M D G Q H W K R L O N D S A
 TTTGAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTCAAAACGACTCGGCA 1200
 Y L A E N G I T A V W I F P A Y K G T S
 TATTTGGCTGAACACGGTATTACTGCGCTGGAATCCCCCGCATATAAGGACGAGC 1260
 Q A D V G Y G A Y D L Y D L G E F H O K
 CAAGCGGATGTTGGGCTACGGTCTTACGACCTTTATGATTAGGGGAGTTTCATCTAAAA 1320
 G T V R T E Y G T E G E L O S A I K S L
 GGGACGGTTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTCCGATCAAAAGTCT 1380
 H S R D I N V Y G D V V I N N E G G A D
 CATTCGCGGACATTAACGTTTACGGGGATGTTGTCATCAACCACAAAGCGCGGCTGAT 1440
 A T E D V T A V E V D F A D R N R V I S
 CGGACCGAAGATGTAACCGCGGTGAAGTCGATCCCGCTGACCGCAACCGGTAATTCA 1500
 G E H L I K A W T H P H F P G R G S T Y
 GGAGAACACCTAATTAAGCCTGACACATTTTCATTTTCGGGGCGCGGACGACATAC 1560
 S D F K W H W Y H P D G T D M D E S R K
 AGCGATTTAAATGGCATTGCTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAG 1620
 L M R I Y K F O G K A N D W E V S H E N
 CTGAACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCAATGAAAAC 1680
 G M Y D Y L N Y A D I D Y D H P D V A A
 GCGAACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCTGATGTCCGACCA 1740

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E I K R W G T W Y A N E L Q L D G F R L
GAAATTAAGAGATGGGGCACTTGGTATGCCAATGAAGTGAATGGACGGTTTCCGTCTT 1800

D A V K N I K F S F L R D W V W N H V R E
GATGCTGTCAAACACATTAAATTTCTTTTTCGGGGATTGGGTTAATCATGTCAGGGA 1860

K T G K E N F T V A E Y W Q N D L G A L
AAAAAGGGGAAGGAAATGTTACGGTAGCTGAATATTGGCAGAATGACTTGGGGCGCTG 1920

E N Y L N K T M F N H S V F D V F L H Y
GAAACTATTTGAACAAAACAAATTTTAATCATTGAGTGTGTGACGTGCCCGCTTCATTAT 1980

Q F H A A S T O G G G Y D N R K L L N G
CAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGT 2040

T V V S E N P L E S V T F V D N H D T Q
ACGGTGGTTTCCAAGCATCCGTTGAAATCGGTTACATTGTGCGATAACCATGATACAG 2100

P G Q S L E S T V Q T W F K P L A Y A F
CCGGGGCAATCGCTTGAGTCGACTGTCAAACATGGTTAAGCGGCTTGCTTACGCTTTT 2160

I L T R E S G Y P O V F Y G D M Y G T E
ATTCTCACAAGGGAATCTGGATACCCCTCAGGTTTCTACGGGGATATGTACGGGACGAA 2220

G D S O R E I P A L K N K I E P I L K A
CGAGACTCCAGCGCGAAATTCCTGCTTGAACACAAAATTGAACCGATCTTAAAGCG 2280

R K Q Y A Y G A Q N D Y F D N H D I V G
AGAAACAGTATCGGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTGGG 2340

W T R E G D S S V A N S G L A A L I T D
TGGACAAGGGGAAGGCGACAGCTCGGTTGCAAAATTCAGGTTTGGCGGCAATTAATAACAGAC 2400

G P G G A K R N Y V G R O N A G E T N H
GGACCCGGTGGGGCAAAGCGAATGTATGTGGCCGGCAAACGCGGTTGAGACATGGCAT 2460

D I T G N R S E P V V I N S E G W G E F
GACATTACCGGAACCGTTCCGAGCCGGTTGTCAATTCGGAAGGCTGGGGAGAGTTT 2520

H V N G G S V S I Y V Q R ***
CACGTAAACGGCGGTCGGTTTCAATTTATGTTCAAGATAGAAGAGCAGAGAGGACGGA 2580

TTTCCTGAAGGAAATCCGTTTTTTTATTTTCCCGCTTATAAAATTTCTTTGATTACATT 2640

TTATAATTAATTTTAAACAAGTGTCAACAGCCCTCAGGAAGGACTTGCTGACAGTTTGA 2700

TCGCATAGGTAAGGCGGGATGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAAT 2760

GTGTCGAAAATGACGGTATCGCGGGTGAATCAATCATCTGAGACTGTGACGGATGAATTG 2820
HINDIII
AAAAAGCTT 2829
*****

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Chart 8. Natural amylase gene sequence.

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BasM1 H E O O E R L Y A R L L T L L F
GGATTCGAATCATGAACAACAAAAACGGCTTACGCCCGATTGCTGACGCTGTTATTTG 60

A L I F L L P H E A A A A A N L N G T L
CGCTCATCTTCTTGGCTGCTCATTCTGCAGCAGCGCGGCAATCTTAATCGGACGCTCA 120
N O Y F E W Y H F M D G O H W R L O N
TGCAGTATTTGAATGGTACATGCCCAATGACGGCCAACTTGGAGCGTTTGCAAAACG 180
D S A Y L A E H G I T A V W I F F A Y K
ACTCGGCATATTTGGCTGAACACGGTATTACTGCCGCTCGGATTCCTCCCGCATATAAGG 240
C T S Q A D V G Y G A Y D L Y D L G E F
GAACGAGCCAAAGCGATGTCGGCTACGGTCTTACGACCTTTATGATTAGGGGAGTTTC 300
N O K G T V R T R Y C T K G E L O S A I
ATCAAAAGGAGCGGTTCCGACAAAGTACGGCAGAAAGGAGAGCTGCAATCTGCGATCA 360
K S L N S R D I N V Y G D V V I M H E K
AAAGTCTTCATTCCTCCGACATTAACGTTTACGGGGATGTGGTCAATCAACCAAAAGCGG 420
G A D A T E D V T A V E V D P A D R H E
CGGCTGATCGGACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCG 480
V I S G E H L I K A N T E F H F P G R G
TAATTTGAGGAGAACCTTAATTAAGCTCGACACATTTTCATTTTCCGGGGCGCGCA 540
S T Y S D F K W H W Y H F D G T D W D E
GCACATACAGCGATTTTAAATGGCATGGTACCATTTTGACGGAACCGATTGGGACGAGT 600
S R E L N R I Y K F Q G K A D W E V S
CCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCA 660
M E N G N Y D Y L N Y A D I D Y D H F D
ATGAAAACGGCAACTATGATTATTGATGTATGCCGACATCGATTATGACCATCCTGATG 720
V A A E I E R M G T W Y A N E L O L D G
TCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAAGTCAATTCGACGGTT 780
F R L D A V K H I K F S F L R D W V H H
TCCGCTTTGATGCTGTCAAACACATTAATTTCTTTTTCGGGGATTGGGTTAATCATG 840
V R E K T G R E N F T V A E Y F Q M D L
TCAGGGAAGAAACGGGGAAGGAAATGTTACGGTAGCTGAATATTGGCAATTCGACTTGG 900
G A L E N Y L N K T N F N L S V F D V P
CGCGCTGGAAACTATTTGAACAAACAAATTTTAATCATTCATGTTTGACGTGCCCGC 960
L N Y O P H A A S T O G C G Y D H R E L
TTCATTATCAGTTCCATGCTGCTGATCGACACAGGGAGGCGGCTF.G' CATGAGGAAATTGC 1020
L N G T V V S K H F L K S Y T F V D N H
TGAACGGTACGGTGGTTTCAAGCATCCGTTGAAATCGTTACATTTGTCGATAACCATG 1080
D T O P G Q S L E S T V Q T W F R P L A
ATACACAGCCGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTAAGCCGCTTGCTT 1140
Y A F I L T R E S G Y F O V F Y G D H Y
ACGCTTTTATCTCACAAGGGAATCTGGATACCTCAGGTTTCTACGGGGATATGTACG 1200
G T E G D S O R E I P A L E N E I E P I
GGACGAAGGAGACTCCGAGCGGAAATTCCTGCTTGAACACAAAATGAACCGATCT 1260
L K A R K O Y A Y C A Q H D Y F D H H
TAAAAGCGAGAAACAGTATCGGTACGGAGCACAGCATGATTATTTGACCACCATGACA 1320
I V G W T R E G D S S V A N S G L A A L
TTGTCCGCTGGACAAGGGAAGGCGACAGCTCGGTTGCAAAATTCAGGTTTGGCGGCAATA 1380

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I T D G P G G A E R H Y V G R O N A G E
TAACAGACGGACCCGGTGGGGCAAGCGAATGTATGTCCGGCCGGCAAAACGCCCGTGAGA 1440

T W R D I T G N R S E P V V I N S E G W
CATGGCATGACATTACCGGAAACCGTTCCGAGCCCGTTGTCAATTCGGAAGGCTGGG 1500

G E F H V N G C S V S I Y V Q R ***
GAGAGTTTCACGTAAACGGCGGGTCCGTTTCAATTTATGTTCAAAGATAGAGAGCAGAG 1560

AGGACGGATTTCCTGAAGGAAATCCGTTTTTTTATTTTGCCCGTCTTATAAATTTCTTTG 1620

ATTACATTTTATAATTAATTTTAACAAAGTGTCAATCAGCCCTCAGGAAGGACTTGCTGAC 1680

AGTTTGAATCCCATAGGTAAAGCGGGGATGAAATGGCAACGTTATCTGATGTAGCAAGA 1740

AAGCAAAATGTGTGAAAAATCAGCGTATCCGGGGTCAATCATCTGAGACTGTGACGG 1800
      BMBI
ATGAATTGAAAAAGCTCCGGATCC 1824
      *****

```

Chart 9. Optimised amylase sequence.

- 30 -

strain gave significant zones of cross-feeding on minimal plates seeded with a phenylalanine auxotroph.

Example 4

5

A semi-synthetic operon construct.

In this example the pheA and aroF genes are combined into a single transcriptional unit. The plasmid pJW2 carries the pheA gene transcribed from the synthetic promoter. The mutant BamHI site downstream of pheA was used to clone the aroF gene carrying a BamHI site in its ribosome binding site. Finally, remaining tyrA sequence was removed by the addition of a synthetic terminator. This plasmid pME202 carries a semi-synthetic operon on an EcoRI-SalI fragment of 2,400 bp. To further increase the utility of this fragment a derivative of pME202 was constructed that carries an EcoRI linker at the SalI site (pME204). Table 3 indicates the approximate numbers found. The plasmid pME202 (ATCC 53136) was introduced into a number of strains and the levels of DAHP synthetase and prephenate dehydratase determined during growth on a number of media. One example of these levels is indicated in Table 1. It is clear that the plasmid causes overproduction of the two activities and a comparison of enzyme levels during growth in the presence and absence of phenylalanine confirms that expression of the two genes has been substantially deregulated.

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Table 3 Plasmid Constructs Using the Phe Operon from pME202 at various copy numbers.

5	Rl_P_****pheA****_****aroF****_T_Sl pME202
	↓ EcoRI linkers cloned at SalI site
10	Rl_P_****pheA****_****aroF****_T_Rl pME204
	Phe operon now carried on EcoRI fragment "cas- sette". This fragment was recloned into the following vectors:
15	
	pBR322 to give pMH19. Copy number approximately 50.
	pLG388 to give pME208. Copy number approximately 10.
	RP4 to give pME214. Copy number approximately 2.
20	(pAT153 to give pME204). Copy number approximately 100.

E.coli HW77 carrying pME202 was tested for L-phe production in cross-feeding experiments (Table 2).
 25 Extremely large zones were obtained in contrast to the untransformed controls. This demonstrates the advantage of being able to coordinate the expression of the two genes in the operon construct. The level of FBI^R aroF product is now acceptable to HW77 growing on minimal
 30 medium.

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Example 5

Utility of the semi-synthetic phenylalanine operon construct in assessing the effect of varying plasmid copy number.

In order to ascertain the effect of copy number on expression the EcoRI fragment from pME204 that carried the aroF pheA operon was recloned into the following vectors: pBR322 (copy number 50) to give pMH19, pLG338 (copy number 10) to give pME208 and RP4 (copy number 1-3) to give pME214. These plasmids were introduced into the tyrA strains HW77 and HW760 and the resultant transformants tested for their growth on minimal medium and L-phe productivity in cross-feeding experiments. The results of these experiments are summarized in Table 4. It was found that while the high copy number derivatives were clearly deleterious to the growth of HW760, HW77 could tolerate all the plasmids. Furthermore, the lower copy derivatives pME208 and pME214 did not prevent the growth of HW760. HW760 carrying pME214 or pME208 gave very large zones of cross-feeding.

This example serves to indicate how the optimum expression level can be attained by varying the copy number of the pheA aroF operon through judicious choice of vector.

Example 6

Extending the host range of the semi-synthetic phenylalanine operon.

The EcoRI fragment from pME204 that encompasses the pheA aroF operon was recloned into a number of broad host range vectors namely:

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RP4 to give pME214
 pCT460 to give pME211
 pKT231 to give pME212

5 Table 4 Growth of HW77 and HW760 Carrying Various Phe Plasmids.

10	PLASMID	GROWTH	
		24 hr	48 hr
	-	++	++++
	pME214	++	++++
	PME208	++	++++
15	pMH19	++	++++
	pME202	++	++++
	pME219	++	++++
	pME170	++	++++
	pME171	++	++++
20	pST35	-	(+)*
	pME237	-	(+)*
	pME238	-	(+)*
	pAT153	++	++++
	-	++	++++
25	pME214	++	++++
	pME208	+	+++
	pMH19	-	+
	pME202	-	(+)
30	* Essentially no growth but a few mutant colonies emerging.		

The strains were grown overnight in L-broth supplemented with suitable antibiotic where appropriate. One microlitre of the overnight culture was then streaked onto minimal agar supplemented with 50 ug

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ml⁻¹ tyrosine and any relevant antibiotic. Growth was scored after 24 hr and 48 hr.

These plasmids were then introduced by transformation, conjugation or mobilization into a variety of strains and L-phe production detected by cross-feeding of an E.coli L-phe auxotroph. Table 5 details the various organism/plasmid combinations and the cross-feeding observed. Significant cross-feeding was observed with a number of organisms including pseudomonads and facultative methylotrophs.

In principle this approach to deregulating a pathway by simply introducing such an artificial operon on a suitable plasmid could be extended to almost any microorganism that had beneficial characteristics. Expression would probably have to be reoptimized, e.g., for Gram positive organisms and this would entail alterations to the synthetic promoter and the various ribosome binding sites. Such an undertaking would be greatly facilitated by the modular structure of the operon and the presence of restriction sites in the Ribosome binding sequences.

Example 7

Extension of the operon - incorporation of the gene for alpha amylase.

The modified alpha amylase gene is flanked by a BamHI site in the Ribosome binding sequence and a BamHI linker in the HindIII site downstream of the gene. The BamHI fragment carrying amy was cloned into the downstream BamHI site in pME202 to give an artificial three gene operon (pPT112) deposited as ATCC 53139, see Chart 11. This plasmid was transformed into HW77 and its growth on starch investigated. A comparison of the growth of HW77/pPT112 on starch and glucose revealed

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that there was little difference between growth rates in starch and glucose, but growth on starch was subject to a much longer lag phase, see figure 2.

5 A fermentation performed on HW77/pPT112 grown on 50g l⁻¹ soluble starch revealed significant accumulation of L-phe (figure 3). Fermentation conditions were as described in example 10 except that glucose was not included in the initial medium and a sugar feed was not used. The soluble starch was batched into the fermenter
10 at 50g l⁻¹ prior to autoclaving.

Table 5 Use of Phe Operon on broad host range plasmids to enhance L-phe production in other genera.

15	Plasmids:	pME202	Phe operon in pAT153 - see text.
		pME204	As pME204 but with <u>EcoRI</u> linker at <u>SalI</u> site.
		pME211	Phe operon from pME204 cloned at <u>EcoRI</u> site of pCT460.
20		pME212	Phe operon from pME204 cloned at <u>EcoRI</u> site of pKT231, transcribed - <u>HindIII</u>
		pME213	Phe operon from pME204 cloned at <u>EcoRI</u> site of pKT231, transcribed -
25			<u>str.</u> Phe operon from pME204 cloned at <u>EcoRI</u> site of RP4, transcribed towards <u>HindIII</u> site.

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-----		HOST				
		pME202	pME214	pME211	pME212	pME213
-----		-----				
5	E.coli	+	+	+	+	+
	E.intermedia	-				
	S.marcesens	-				
	S.typhimurium	+				
	Ps.fluorescens	-		+	+	+
10	Ps.putida			+	+	+
	Facultative Methylophils: (grown on methanol)					
	Mycoplasma rubra		+			
	Pseudomonas spp.		+			
	Methylobacterium					
15	organophilum		-			
	Ps.extorquens		-			
	Vibrio extorquens		-			

20 + Production of L-Phe caused by introduction of plasmid as detected by cross-feeding experiments or by TLC analysis of shake flask media.

- No L-Phe production detected in response to plasmid. Remaining host/plasmid combinations were not tested.

25 Example 8

Extension of the operon - Incorporation of the aspC gene.

30 The artificial pheA aroF operon was further modified to include the gene for aspartate amino transferase (aspC) as follows: pIF18 which carries the aspC gene flanked by a BamHI site in the ribosome binding site and a BglII site downstream of the gene was used as

35 a source of the BamHI-BglII fragment encompassing aspC. This fragment was then cloned into pME202 that

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had been partially cleaved with BamHI to give a derivative pME219 (deposited as ATCC 53138) that carries the aspC gene downstream of aroF so as to yield a three gene artificial operon (chart 10). When introduced into HW77 this plasmid caused L-phe production as detected by cross-feeding. Analysis of cell free extracts revealed elevated levels of aspC activity (Table 6).

Table 6 Phenotypic Effect of pME219.

pME219 was transformed into HW77 to give HW1058.

Strain	Plasmid	Aspartate Aminotransferase Activity U mg ⁻¹
HW77	-	604
HW1058	pME219	1913

The strains were grown to O.D.₆₀₀ in glucose minimal medium supplemented with 0.5% casamino acids.

The benefit of this construct is that the plasmid not only deregulates the phenylalanine pathway but also yields elevated aspC levels. This has utility in that an L-phe fermentation can be supplemented with chemically synthesized PPA along with aspartate or glutamate to increase L-phenylalanine titres. Such a hybrid fermentation/bioconversion has advantages that include greater ease of L-phe isolation and higher L-phe productivity.

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GAATTCCTTTTGTGACAGCGTGAACAGTACCGGTATAAATACTAAAGTCACAAAAAG 60
 N T S E N S L L A L E E E I S A L
 GCAACACTATGACATCGGAACCCGTTACTGCGCGTCCGAGAGAAAATCAGCGCGGTGG 120
 D E K L L A L L A E E R R E L A V E V G E
 ATGAAAAATTATAGCGTTACTGCGAGAACCGCGGAACCGCGTCCAGGTGGGAAAAAG 180
 A K L L S E R R P V R D I D R E R D L L E
 CCAAACTGCTCTGCGATCGCCCGTACGTGATATGATCTGAAACCGCATTTGCTGGAAA 240
 R L I T L G R A E R L D A N Y I T R L F
 GATTAAATACCGCTCGGTAAAGCGCACCATCTGGACGCCCATACATTACTCGCGCTGTCC 300
 G L I I E D S V L T Q Q A L L Q O N L N
 AGCTCATCATTGAAGATTCGGTATTAAGTACGACGCGTTTGTCTCCAACACATCTCAATA 360
 K I N P H S A R I A P L G P H G S Y S N
 AAATTAATTCGCACTCAGCAGCGCATCGCTTTCTCGGGCCCAAGGTTCTTATTCCTC 420
 L A A R O Y A A R H P H O P I E S G C A
 TTGGCGCGCGCCGATATGCTGCGCGTCACTTTGAGCAATTCATTGAAAGTGGCTGGCCA 480
 K F A D I F N Q V E T G Q A D Y A V V P
 AATTTGCGCATATTTTAACTAGGTGGAACCGCGCCAGCGCGCATATCGCGTCTGACCGA 540
 I E N T S S G A I N D V Y D L L O N T S
 TTGAAAAATACGAGCTCGGTGCGCAAAACGACGTTTACGATCTGCTGCAACATACGAGT 600
 L S I V G E N T L T I D H C L L V S G T
 TGTGATTTGTTGGCGAGTACGTTAAGTATCGACCATTTGTTGTTGCTCTCGCGCACTA 660
 T D L S T I N T V Y S E P Q P P Q Q C S
 CTGATTATTCACCATCAATACGGTCTACAGCGCATTCGCGAGCCATTCCAGCAATGCGACA 720
 K F L N R Y P H W K I E Y T E S T S A A
 AATTCCTTAATCTTATCGCGCATGGAAGATTGAATAACCGAAAGTACGCTTGGCGCAA 780
 H E E V A Q A K S P H V A A L G S E A G
 TGGAAAAGGTTGCACAGCGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCG 840
 G T L Y G L O V L E R I E A N Q R O N P
 GCACTTTGTACGGTTTCAGGTACTGGAGCGTATTGAAGCAATCAGCGACAAAATCTCA 900
 T R V V V L A R K A I N V S D Q V P A E
 CCGGATTTGTGGTGTGGCGCGTAAAGCCATTAAAGTGTCTGATCAGGTTCCCGCGAAAA 960
 T T L E N A T G Q Q A G A L V E A L L V
 CCAGGTTGTAAATGCGGACCGCGCAACAGCGGTGCGGTGGAAGCGTTGCTGGTAC 1020
 L R N E N L I N T R L E S E P I N G N P
 TCGGCAACCACAATCTGATTATGACCGCTCTGGAATCAGCGCCGATTACGGTAATCCAT 1080
 W R S P W E E N F Y L D I Q A N L E S A
 CGAGATCTCCATGGGAAGAGATGTTCTATCTGGATATTAGGCCAATCTTGAATCAGCGG 1140
 E H Q K A L E E L G E I T R S N K V L G
 AAATGCAAAAAGCATTGAAGAGATTAGCGGAAATCACCGTTCAATGAAGGATTGGGCT 1200
 C Y P S E N V V P V D F T N
 GTTACCCAGTGAAGACGTAGTGCTGTGATCCAACTGATGAAGAGGATCCCCATCAT 1260
 Q K D A L N H V E I T D S Q V L N T F E
 GCAAAAAGACCGCGTGAATAACGTACATATTACCGACGAACAGGTTTAAATGACTCCGA 1320
 O L K A A P F L S L Q Q E A Q I A D S E
 ACAACTGAAGCCCGCTTTTCCATTGAGCGCTGCAACAAGAGCCCGAGATTGCTGACTCGG 1380
 K S I S D I I A G R D P R L L V V C G P
 TAAAGCATTTCAGATATTATCGCCGCGCGCGATCTCTGCTGCTGGTAGTATGTTGGTCC 1440
 C S I N D P E T A L E Y A R R F K A L A
 TTGTTCCATTATGATCCGGAACGCTCTGGAATATGCTGCTGATTAAGCCCTTCC 1500
 A E V S D S L Y L V R V Y P E E P T
 CCGCAGGTCAGCGATAGCCCTCTATCTGGTAATGCGCGCTCTATTTGAAAAACCGGTAC 1560

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T V G W K G L I N D F N H D G S F D V E
 CACTGTCCGGCTCGAAAGCGTTAATTAACGATCCCAZATCGATCGCTCTTTTGTATGEAGA 1620
 A G L Q I A R E L L L L E L V N H G L F L
 AGCCGGGCTGCAGATCGCCGCTAAATTCCTGCTTGAGCTGGTGAATATGGGACTGCCACT 1680
 A T E A L D P N S P O Y L G D L F S W S
 GCGACCGGAAGCGTTAGATCCGAATAGCCCGCAATACCTGGGCGATCTGTTAGCTGGTC 1740
 A I G A R T T E S O T H E R E N A S G L S
 AGCAATTGGTGTCTCGZACAACCGAATCGCAAACTCACCGTGAATGGCTCTCGGGCTTTC 1800
 H P V G F K N G T D G S L A T A I N A M
 CATGCCGGTGGTTTAAAAACGGCACCGACGGCAGTCTGGCAACAGCAATTAACGCTAT 1860
 R A A A O P E R F V G I N Q A G O V A L
 GCGCGCGCGCGCCAGCCCGCACCGTTTTTGTGGCATTAAACAGGCGAGGCGAGGTTGCGTT 1920
 L Q T O G N P D G E V I L E G G K A P H
 GCTACAACTCAGGGGAATCCGGACGGCATGTGATCTCGCGCGTGGTAAAGCGCGCA 1980
 Y S P A D V A Q C E K E N E Q A G L R P
 CTATAGCCCTCGCGATGTTGGCAATGTGAAAAAGAGATGGAACAGGCGGACTGCGCCC 2040
 E L N V D C S E G N S N E D Y R R O P A
 GTCTGTGATGGTAGATTGCAGCCACCGTAATTCATAAAGATTATCGCGCTCAGCGCTGC 2100
 V A E S V V A Q I K D G N R S I I G L N
 GGTGGCAGAAATCCGTGGTTGCTAAATCAAGATGGCAATCGCTCAATTATTGGTCTGAT 2160
 I E S N I E G N Q S S E Q P R S E M K
 GATCGAAAGTAATATCCAGGAGGGCAATCAGTCTTCCGAGCAACCGCGCAGTGAATGAA 2220
 Y G V S V T D A C I S W E N T D A L L R
 ATACGGTGTATCCGTAAACCGATGCTTCATTAGCTGGGAAATGACCGATGCTTGTGCG 2280
 E I N O D L H G Q L T A R V A *****
 TGAATTTCATCAGGATCTGAACGGGCGAGTGAACGGCTCGCGTGGCTTAATGAGGATCCTC 2340
 H F E N I T A A P A D F I L G L A D L
 GTCATGTTTGAGAACATTACCGCGCTCTGCGGACCGGATTCTGGGCTGGCGGATCTG 2400
 F R A D E R P G K I N L G I G V Y K D E
 TTTCTGCGCGATGAACGTCCCGGCAAAATTAACCTGGGATGGTGTCTATAAGATGAG 2460
 T G K T P V L T S V K K A E O Y L L E N
 ACGGGCAAAACCCGGTACTGACAGCGTGAAAAAGGCTGAACAGTATCTGCTCGAAAT 2520
 E T T K N Y L C I D G I F E F G R C T Q
 GAAACCAACAAAAATTACCTCGGCATTGACGGCATCCCTGAATTGGTGGCTGCACCTCAG 2580
 E L L F G K G S A L I N D K R A R T A Q
 GAACTGCTGTTTGGTAAAGGTAGCGCCCTGATCAATGACAAACGTGCTCGCACGGCACAG 2640
 T P G G T G A L R V A A D F L A K N T S
 ACTCGGGGGGCACTGGCGCACTACGCTGGCTGGCGATTCTCGGCAAAAAATACCAGC 2700
 V K R V W V S N P S W P N E K S V F N S
 GTTAAGCGTGTGGGTGAGCAACCCAAGCTGGCGCAACCATAGAGCGCTCTTTAACTCT 2760
 A G L E V R E Y A Y Y D A E N H T L D F
 GCAGGTCTGGAAGTTCTGTAATACGCTTATTATGATCGGGAATACACTCTTGACTTC 2820
 D A L I N S L N E A Q A G D V V L F E G
 GATGCACTGATTACAGCCTGAATGAAGCTCAGGCTGGCGACGTAGTGTGTTCCATGGC 2880
 C C E N F T G I D F T L E O W O T L A Q
 TGCTGCCATAACCCCAACCGGTATCGACCTACGCTGGAACAAATGGCAACACTGGCACAA 2940
 L S V E R G W L P L F D F A Y O G F A E
 CTCTCGTTGAGAAAGGCTGGTACCCTGTTTGACTTCGCTTACCAGGGTTTTGCCCGT 3000
 G L E E D A E G L R A P A A M H K E L I
 GGTCTGGAAGAAGATGCTGAAGGACTCGCGCTTTCCCGCTATGCATAAAGAGCTGATT 3060
 V A S S Y S K N F G L Y N E R V G A C T
 GTTGCAGTTCCTACTCTAAAACTTTGGCTGTACAAAGAGCGTGTGTGGCGCTGTACT 3120

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L V A A D S E T V D R A F S O N K A A I
 CTGGTTGCTGCCGACAGTGAACCGTTGATCGCCATTGAGCCAAATGAAGCGCGGATT 3180
 R A N Y E M P P A N G A S V V A T I L S
 CCGGCTAACTACTCTAACCACACGACACGGCGCTTCTGTTGTTGCCACCATCTTGAGC 3240
 N D A L R A I W E O E L T D M R O R I O
 AAGGATGCGTTACGTGCGATTGGGAACAAGAGCTGACTGATATGCCGCCAGCGTATTGAG 3300
 R M R O L F V N T L O E K G A M R D F S
 CGTATGCGTCAGTTGTTCTGTCATACGCTGCAAGGAAAAGCGCAACCGCGACTTCAGC 3360
 F I I K O N G N F S P E G L T E E Q V L
 TTATCATCAACAGAACCGCATGTTCTCTTCAGTGGCGCTGACAAAAGAACAAAGTGCTG 3420
 R L R E E F G V Y A V A S G R V N V A G
 CGTCTGCGCGAAGAGTTTGGCGTATATGCGGTTGCTTCTGTTGCGCTAAATGTGGCCGGG 3480
 N T F D N N A F L C E A I V A V L ***
 ATGACACCAGATAACATGGCTCCGCTGTGCGAAGCGATTGTGGCAGTGTCTGAAGCATT 3540
 AAAACAATGAAGCCCGCTGAAAAGCGGGCTGAGACTGATGACAAACGCAACATTGCCTGA 3600
 TGGCGTACGCTTATCAGGGAGATCCCGGGCGATTCTACGCCCGGGTTTTTATGTGAC 3659
 X-----

Chart 10. Sequence of the pheA aroF aspC
operon in pME219.

KEY

Hybrid BglIII/BamHI site downstream of asnC

X-----

- 41 -

GAATTCCTTTTGTGTGACAGCGTGAACAGTACGGGTATAATACTAAAGTCACAAAAG 60
N T S E N F L L A L R E E I S A L D
GCAACACTATGACATCGGAAACCCGTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGG 120
E K L L A L L A E R R E L A V E V G K A
ATGAAAAATTATTAGCGTTACTGGCAGAACGGCGGAAGTGGCGCTCGAGGTGGGAAAAG 180
K L L S H R F V R D I D R E R D L L E R
CCAAACTGCTCTCGCATCGCCCGTACGTGATTTGATCGTGAACGCGATTGCTGGAAA 240
L I T L G K A H N L D A N Y I T R L F Q
GATTAATTACGCTCGGTAAAGCGCACCATCTGGACGCCATTACATTACTCGCCTGTTC 300
L I I E D S V L T Q Q A L L Q Q H L N R
AGCTCATCATGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATA 360
I N F H S A R I A F L G P K G S Y S H L
AAATTAATCCGCACTCAGCAGCGCATCGCTTTCTCGGCCCAAGGTCTTATTCCTCATC 420
A A R O Y A A A R H F E Q F I E S G C A E
TTGCGGCGCGCGCAGTATGCTGCCCGTCACTTTGAGCAATTCAATGAAGTGGCTGCGCCA 480
F A D I F N O V E T G Q A D Y A V V P I
AATTGCGGATATTTTAATCAGGTGGAACCGGCCAGGCCGACTATGCCGCTGTACCGA 540
E N T S S G A I N D V Y D L L Q H T S L
TTGAAAATACAGCTCCGGTGCATAAACGACGTTTACGATCTGCTGCAACATACCAGCT 600
S I V G E N T L T I D H C L L V S G T T
TGTGATTTGTTGGCGAGATGACGTTAACTATCGACCATTTGTTGTTGGTCTCCGCGACTA 660
D L S T I N T V Y S H F O F F O O C S E
CTGATTTATCCACCATCAATACGGTCTACAGCCATCCGCGCCATTCCAGCAATGCAGCA 720
F L N R Y F H W E I E Y T E S T S A A H
AATTCCTTAATCGTTATCCGCACTGGAAGATTGAATATACGAAAGTACGTCTGCGGCCA 780
E K V A Q A K S P H V A A L G S E A G G
TGGAAAAGTTGCAAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCG 840
T L Y G L Q V L E R I E A N Q R O N F T
GCATTTGTACGGTTTGCAGGTACTGGAGCGTATTGAAGCAATCAGCGCAAAAACCTCA 900
R F V V L A R K A I N V S D Q V P A K T
CCCGATTGTGGTGTGGCGCGTAAAGCCATTAACTGTCTGTATCAGGTTCCGCGGAAAA 960
T L L H A T G Q Q A G A L V E A L L V L
CCACGTTGTTAATGGCGACCGGGCAACAGCGGTGCGCTGGTTGAAGCGTTGCTGGTAC 1020
R N H N L I M T R L E S R P I N G N P W
TGGCAACCAATCTGATTATGACCCGCTCTGGAATCAGCGCCGATTACGGTAATCCAT 1080
R S P W E E N F Y L D I O A N L E S A E
GGAGATCTCCATGGGAAGAGATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCG 1140
H Q K A L K E L G E I T R S H E V L G C
AAATGCAAAAAGCATTGAAGAGTTAGGGGAAATCACCGTTCAATGAAGGTATTGGGCT 1200
Y P S E N V V P V D P T * H
GTTACCCAAGTGAGAACGTAGTGCTGTTGATCCAACCTGATGAAAAGGATCCCCATCAT 1260
Q K D A L N M V H I T D E Q V L N T P E
GCAAAAAGACCGCTGAATAACGTACATATTACCGAGCAACAGGTTTAAATGACTCCGGA 1320
Q L K A A F P L S L Q Q E A Q I A D S R
ACAACTGAAGGCGCTTTTCCATTGAGCTGCAACAAGAAGCCAGATTGCTGACTCGCG 1380
K S I S D I I A G R D P R L L V V C G P
TAAAGCATTTTCAGATATTATCGCCGGGCGCGATCTCGTCTGCTGGTAGTATGTGCTCC 1440
C S I H D P E T A L E Y A R R P R A L A
TTGTTCCATTGATATCCGAAACTGCTCTGGAATATGCTCGTCAATTAAGCCCTTGC 1500
A E V S D S L Y L V M R V Y F E R P R T
CCGAGAGGTGAGCATAGCCTCTATCTGGTAATGCGCGTCTATTTTGAAAAACCCCGTAC 1560

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T V G W E G L I N D P H N D G S F D V E
CACTGTGGCTGGAAAGGGTTAATTAACGATCCCCATATCGATGGCTCTTTTGATGTAGA 1620

A G L O I A R K L L L L L V N M G L P L
AGCCGGGCTGCAGATCGCGCGTAAATGTGCTTGAGCTGGTGAATATGGGACTGCCACT 1680

A T E A L D P H S F O Y L G D L F S M S
CGCGACCGAAGCGTTAGATCCGAATAGCCCGCAATACCTGGGCGATCTGTTTAGCTGGTC 1740

A I G A R T T E S O T H R E M A S G L S
AGCAATGGTGTCTGTACAAACGAATCGCAAACTCACCGTGAAATGGCCTCCGGGCTTTC 1800

N F V G F E N G T D G S L A T A I N A M
CATGCCGGTGGTTTTAAAAACGGCACCGGCGGCTCTGGCAACAGCAATTAACGCTAT 1860

R A A A Q P H R F V G I H Q A G Q V A L
CG 1920

L Q T Q G N P D G H V I L R G G K A P H
CGTACAACTCAGGGGAATCGGACCGCGCATGTGATCTCGCGCGTGGTAAAGCGCGCGAA 1980

Y S P A D V A Q C E E E H E O A G L R F
CTATAGCCCTCGCGGATGTTGCGCAATGTGAAAAGAGATGGAACAGCGCGGACTCGCGCC 2040

S L H V D C S H G N S H R D Y R R O P A
GTCTCTGATGGTAGATTGCGCGCGCGTAAATCCAAATAAGATTATCGCGCTCAGCGTGC 2100

V A E S V V A Q I K D G M R S I I G L N
GGTGGCAGAAATCCGTGCTTGTCTCAATCAAGATGGCAATCGCTCAATTATGCTCTGAT 2160

I E S N I N E G N Q S S E O P R S E M K
GATCGAAGTAATATCCAGCGCGCGCAATCAGTCTTCCGAGCAACCGCGCGAGTGAATGAA 2220

Y G V S V T D A C I S W E N T D A L L R
ATACGGTGTATCGTAACCGATGCGCTGCATTAGCTGGGAAATGACCGATGCGCTTGCCTGC 2280

E I N O D L N G O L T A R V A . . .
TGAAATTCATCAGGATCTGAACCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 2340

N K Q Q E R L Y A R L L T L L F A L I
ATCATGAACCAACAAAACGGCTTTACGCGCGGATTGCTGACGCTGTTATTGCGCTCATC 2400

F L L P H S A A A A A A N L N G T L N O Y
TTCTTGCTGCTTCTTCTGCGAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG 2460

F E W Y N P N D G Q N H K R L O N D S A
TTTGAATGGTACATGCGCAATGACCGCGCAACATTGGAAGCGTTTGCAAAACGACTCGGCA 2520

Y L A E N G I T A V W I P P A Y E G T S
TATTTGGCTGAACACCGGTATTAATGCGCTGCGATTCCTCCCGCGCATATAAGGGAACGAGC 2580

Q A D V G Y G A Y D L Y D L G E F H Q K
CAAGCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTAGGGGAGTTTCATCAAAAA 2640

G T V R T N Y G T K C E L O S A I K S L
GGGACCGGTTCCGACAAAGTACGGCACAAAGGAGAGCTGCAATCTGGCATCAAAAGTCTT 2700

N S R D I N V Y G D V V I N H K G C A D
CATTCGCGCGCATTAACGTTTACGGCGATGTGGTCAACACCAAAAGCGCGCGCTGAT 2760

A T E D V T A V E V D P A D R N R V I S
CGGACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTC 2820

G E N L I K A N T H P F P G R G S T Y
GGAGAACACCTAATTAAGCGCTGGACACATTTTCATTTTCCGGGGCGCGCGAGCACATAC 2880

S D F E W N W Y N F D G T D W D E S R K
AGCGATTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAG 2940

L M R I Y E F O G K A W D W E V S H E N
CTGAACCGCATCTATAAGTTTCAAGGAAAGCTTGGGATGGGAAGTTTCCAATGAAGAA 3000

G N Y D Y L N Y A D I D Y D H P D V A A
GGCAACTATGATTATTTGATGTATGCGGACATCGATTATGACCATCTGATGTGCGAGCA 3060

E I K R W G T W Y A N E L O L D G F R L
GAAATTAAGAGATGGGCGACTTGGTATGCCAATGAACTGCAATGCGCGGTTTCCGTCTT 3120

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D A V K N I K P S F L R D W V M H V R E
 GATGCTGTCAAACACATTAAATTTCTTTTTTCCGGGATTGGGTAAATCATGTCAGGGA 3180
 K T G K E N F T V A E Y W Q N D L G A L
 AAAACGGGAAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGGCTG 3240
 E N Y L N K T N F N H S V F D V P L E Y
 GAAACTATTTGAACAAAACAAATTTTAATCATTCAAGTGTGACGTCGCCGCTTCATTAT 3300
 Q F N A A S T Q G G G Y D H R K L L N G
 CAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATGCTGAACGGT 3360
 T V V S K N F L K S V T F V D N N D T Q
 ACCGTCGTTTCCAAGCATCCGTTGAAATCGGTACATTGTCGATAACCATGATACACAG 3420
 F G Q S L E S T V Q T W F K P L A Y A F
 CCGGGCAATCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTT 3480
 I L T R E S G Y P Q V F Y G D H Y G T K
 ATTCTCACAGGGAATCTGGATACCCCTCAGGTTTTCTACGGGGATATGTACGGGACGAA 3540
 G D S O R E I F A L K H K I E F I L K A
 GGAGACTCCAGCGCGAAATTCCTGCTTGAACACAAAATTGAACCGATCTTAAAGCG 3600
 R K Q Y A Y G A Q H D Y F D H H D I V G
 AGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTGGC 3660
 W T R E G D S S V A N S G L A A L I T D
 TGGACAAGGGAAGGCGACAGCTCGGTTGCAAAATTCAGGTTTGGCGGCATTAAACAGAC 3720
 G F G G A K R M Y V G R O N A G E T W H
 GGACCCGCTGGGGCAAAGCGAATGTATGTCGGCCGGCAAACGCCGCTGAGACATGGCAT 3780
 D I T G N R S E P V V I M S E G W G E F
 GACATTACCGGAAACCGTTCCGAGCCGGTTGTCAATTCGGAAGGCTGGGAGAGTTT 3840
 H V N G G S V S I Y V O R *
 CACGTAAACGGCGGCTCGGTTTCAATTTATGTTCAAAGATAGAAGAGCAGAGAGGACGA 3900
 TTTCTGAAGGAAATCCGTTTTTATTTTCCCGCTTATAAATTTCTTTGATTACATT 3960
 TTATAATTAAATTTTAAACAAAGTGTATCAGCCCTCAGGAAGGACTTGTGACAGTTTGA 4020
 TCGCATAGGTAAGCCGGGATGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAT 4080
 GTGTGAAAATGACCGTATCCCGGCTGATCAATCATCTGAGACTGTGACGGATGAATTG 4160
 AAAAGCTCCGGATCCCGGCGATTCTACGCCCGGTTTTTTATGTGAC 4150

Chart 11. Sequence of the pheA aroF amy operon
in pPT112.

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Example 9

Application of phenylalanine operon plasmids to strain screening.

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A total of 31 strains of E.coli, along with some other enteric species, were transformed with pME202 and screened for both growth on minimal medium and cross-feeding of an L-phe auxotroph. The results are summarized in figure 4. Since the complete deregulation of pheA and aroF would take 5-6 rounds of conventional mutagenesis it can be seen that the composite plasmid approach has a very great advantage in terms of speed. The mutagenesis of 45 strains to this extent would conservatively take ten man years to accomplish, and there would be no guarantee that the lesions would be directly comparable. Using the composite plasmid approach strains can easily be deregulated with identical lesions and put through initial screens in under two weeks.

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Example 10

Utility of Phe Plasmids in Fermentation...

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To examine the potential of strains carrying Phe plasmids for L-phenylalanine production we compared the productivity of HW1057 (HW77pME202) with HW77 in 10 liter fermenters.

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Both strains were grown from a single colony in L-broth for 15 hr. The HW1057 culture was supplemented with 100 μ g ampicillin ml^{-1} . These overnight cultures were used to inoculate a shake flask containing the following minimal medium:

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	Glucose	19.4
	(NH ₄) ₂ HPO ₄	2.0
	K ₂ PO ₄	6.6
	FeAmmCit	0.1
5	Tyrosine	0.225
	MgSO ₄ ·7H ₂ O	2.25

Rates of addition are given in grams per liter. Trace element solution was also added (1 ml per liter). Ampicillin was added to the medium for the growth of HW77pME202.

This seed culture was grown for 12 hr at 33° and then used to inoculate a 10 liter stirred tank fermenter containing the same medium. The fermentation was continued until the tyrosine was exhausted, i.e., about 11 hr at which point the glucose feed was commenced. Growth was limited by the tyrosine starvation to an A₆₇₀ of about 20. A 70% glucose feed was used at a rate of 0.63 g l⁻¹hr⁻¹. L-phenylalanine concentrations were monitored throughout the fermentation.

Figure 5 gives details of a typical comparison between HW77 and HW77 carrying the Phe plasmid pME202. It is clear that the presence of the plasmid has enhanced both the rate of accumulation and the final broth titre of L-phenylalanine. The details of productivity and final broth titre are summarized in the following table:

	STRAIN	PRODUCTIVITY (gm/Liter, hr)	FINAL TITRE (gm/Liter, hr)
30	-----	-----	-----
	HW77	0.11	6.07
	HW77+pME202	0.43	11.6
	-----	-----	-----

Analysis of the fermenter broths also revealed that whereas HW77 accumulated significant quantities of

- 46 -

both prephenate and chorismate HW77pME202 did not. This indicates that the deregulated pheA gene on pME202 is having the desired effect of increasing the flux down the L-phenylalanine branch of the pathway by elevating prephenate dehydratase activity in vivo.

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CLAIMS:

1. As a composition of matter, a composite plasmid comprising:

(a) a first DNA segment containing a replicon covalently joined to a second DNA segment containing one or more transcriptional units; (b) if only one transcriptional unit, then an artificial operon containing two or more genes coding for two or more polypeptides active in the synthesis of an amino acid; (c) if two or more transcriptional units, then a first transcriptional unit comprising an artificial operon containing one or more genes coding for one or more polypeptides active in the synthesis of an amino acid and one or more additional transcriptional units containing one or more genes coding for polypeptides useful in the production of an amino acid.

2. The composite plasmid of Claim 1(c) wherein an additional transcriptional unit contains one or more genes coding for one or more catabolic enzymes or a transport protein.

3. The composite plasmid of Claim 2 wherein a catabolic enzyme is an amylase.

4. The composite plasmid of Claim 1 wherein the polypeptides active in the synthesis of an amino acid are enzymes.

5. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of phenylalanine is pheA.

6. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tryptophan, phenylalanine or tyrosine is aroF.

7. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tyrosine is tyrA.

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8. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of phenylalanine or tyrosine is tyrB.

5 9. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tryptophan, phenylalanine or tyrosine is aroL.

10 10. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tryptophan, phenylalanine or tyrosine is aroH.

15 11. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tryptophan, phenylalanine or tyrosine is aroG.

12. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of tryptophan is trpE.

20 13. The composite plasmid of Claim 4 wherein one of the genes coding for an enzyme active in the synthesis of phenylalanine or tyrosine is aspC.

14. The composite plasmid of Claim 4 wherein one of the genes coding for enzymes active in the synthesis of tryptophan is trpB, trpA, trpD, or trpC.

25 15. The operon plasmid of Claim 1 wherein the replicon is capable of replication in a prokaryotic cell.

16. A method of producing a composite plasmid comprising:

30 (a) mutating individually to feedback inhibition resistance one or more genes coding for an enzyme active in the synthesis of an amino acid; (b) connecting the mutated genes to a transcriptional unit; (c) expressing the transcriptional unit resulting in enzyme
35 synthesis thereby increasing the synthesis of the amino acid.

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17. The method of Claim 16 wherein an additional step comprises:

(a) inserting an additional transcriptional unit containing one or more genes coding for one or more enzymes useful in the production of amino acids.

18. A method of screening for microorganisms useful in amino acid production comprising:

(a) transforming a bacterial cell with a plasmid containing an artificial operon containing a gene coding for the synthesis of a feedback resistant enzyme that is rate-limiting in amino acid synthesis;

(b) selecting the transformed cells;

(c) isolating a pure culture;

(d) quantitating the production of the desired amino acid or an amino acid precursor from the pure culture in (c).

19. The method of Claim 18 wherein an additional step comprises:

(e) cross-feeding to determine one or more of the following: (1) amino acid concentration, (2) amino acid precursor concentration, or (3) growth in the presence of an amino acid analog.

20. A composite plasmid of Claim 1 comprising plasmid pME202.

21. A composite plasmid of Claim 1 comprising plasmid pME214.

22. A composite plasmid of Claim 1 comprising plasmid pME219.

23. A composite plasmid of Claim 1 comprising plasmid pPT112.

24. A method for the production of an amino acid comprising: (a) fermenting a bacterial cell containing the composite plasmid of claim 1; (b) isolating the amino acid.

25. The method of Claim 24 wherein the amino acid is an aromatic amino acid.

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26. The method of Claim 25 wherein the aromatic amino acid is L-phenylalanine.

27. The method of Claim 25 wherein the aromatic amino acid is tyrosine.

5 28. The method of Claim 25 wherein the aromatic amino acid is tryptophan.

29. A composition of matter comprising a DNA sequence selected from the following:

- 10 (a) chart 3;
(b) chart 5;
(c) chart 7;
(d) chart 9;
(e) chart 10;
(f) chart 11.

15 30. A plasmid containing a DNA sequence of claim 29.

31. A method for the production of an aromatic amino acid comprising:

- 20 (a) fermenting a bacterial cell containing a plasmid of claim 30;
(b) isolating the amino acid.

32. The method of claim 32 wherein the aromatic amino acid is L-phenylalanine.

25 33. The method of claim 32 wherein the aromatic amino acid is L-tyrosine.

34. The method of claim 32 wherein the aromatic amino acid is L-tryptophan.

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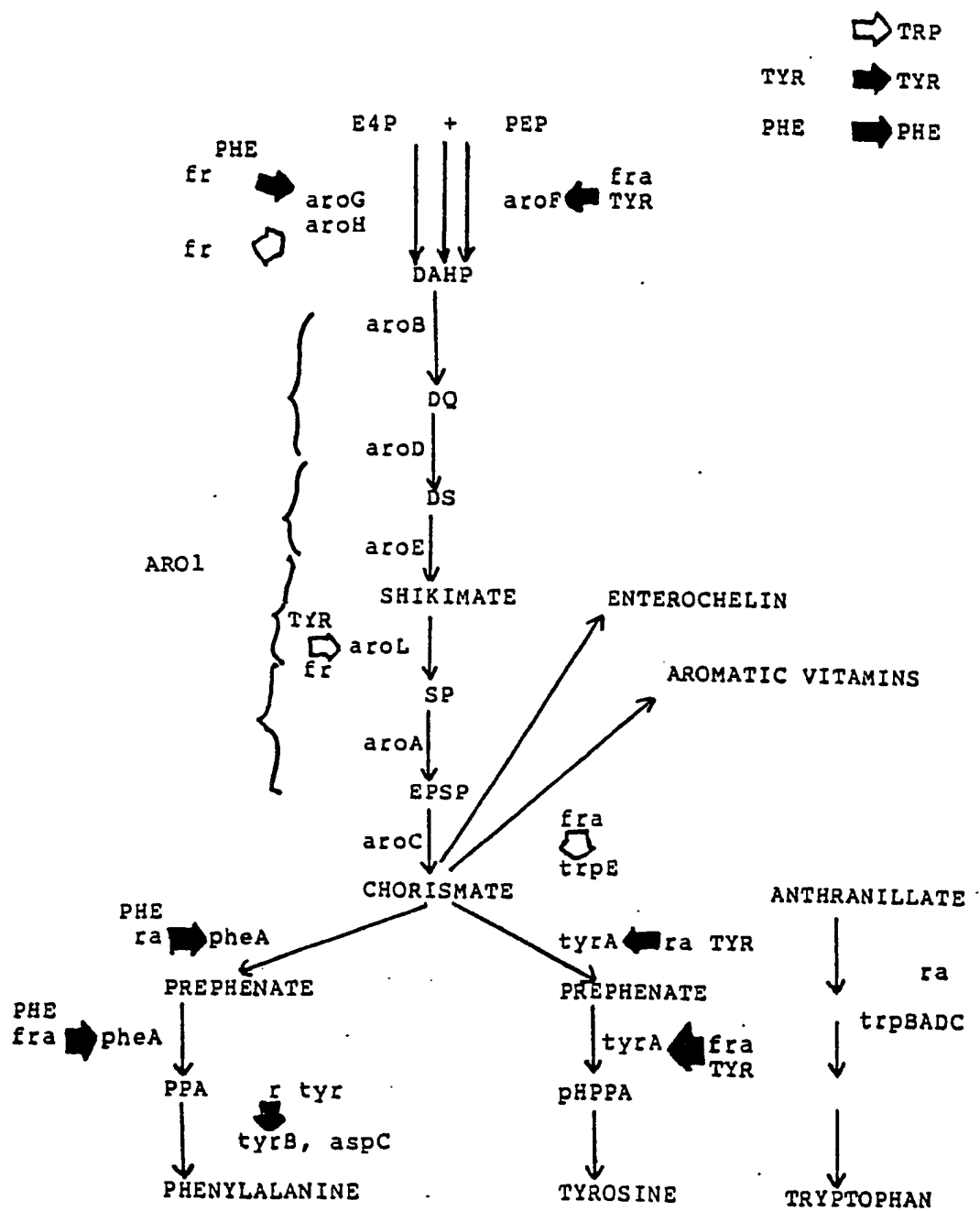


FIG.1

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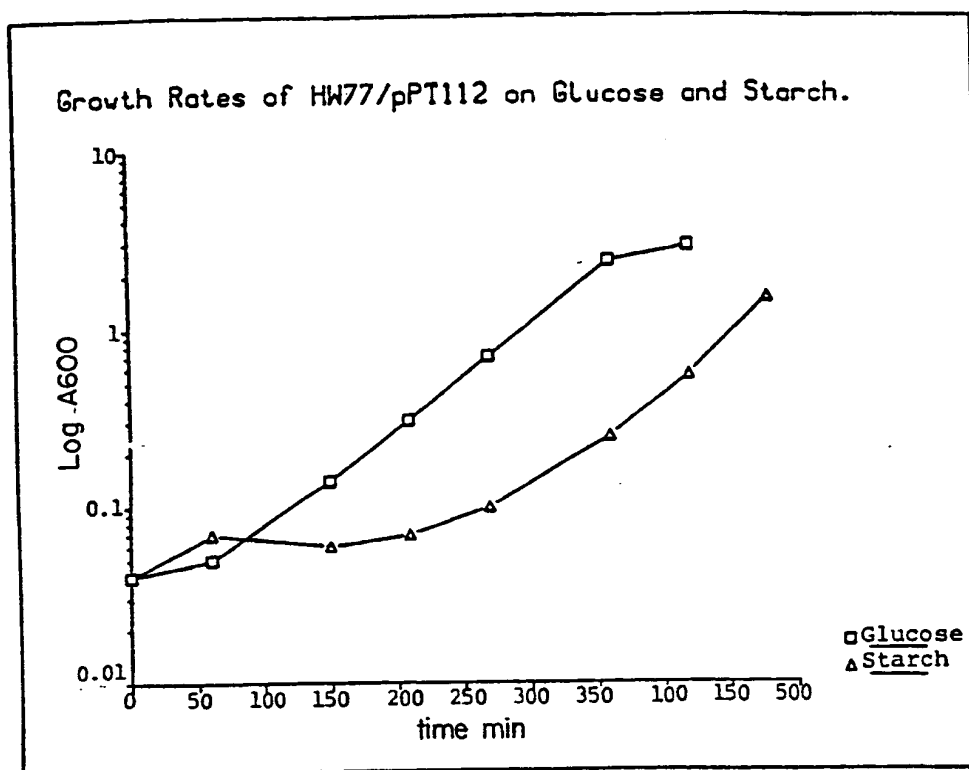


FIG.2

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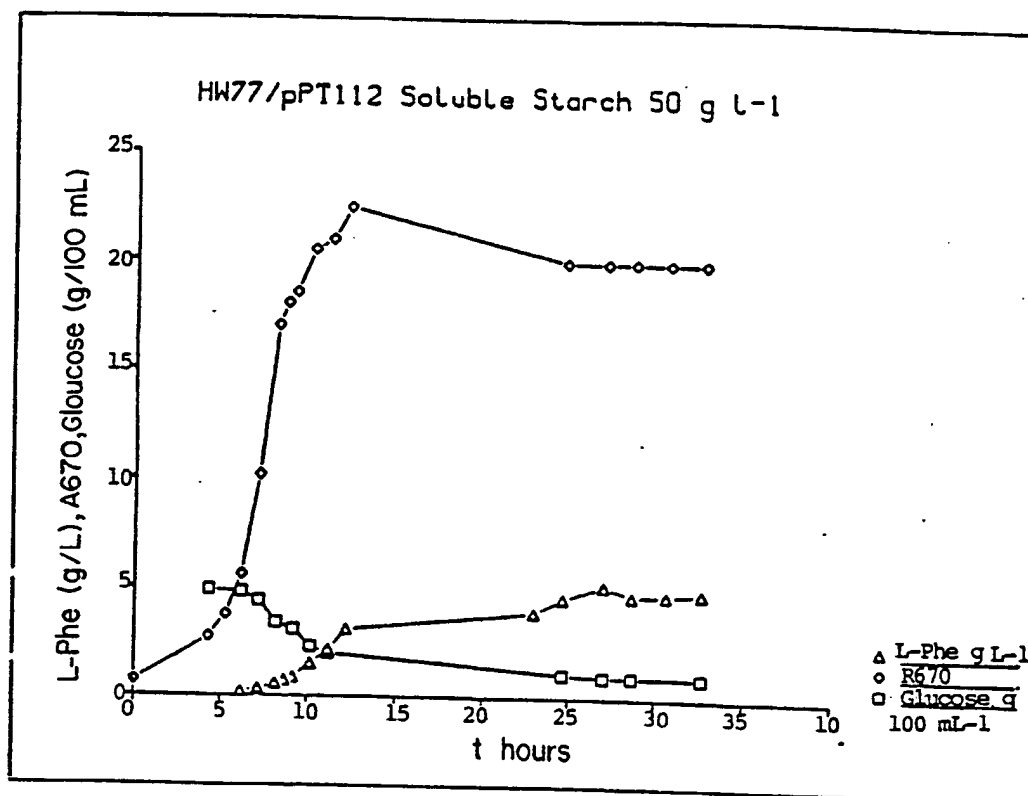
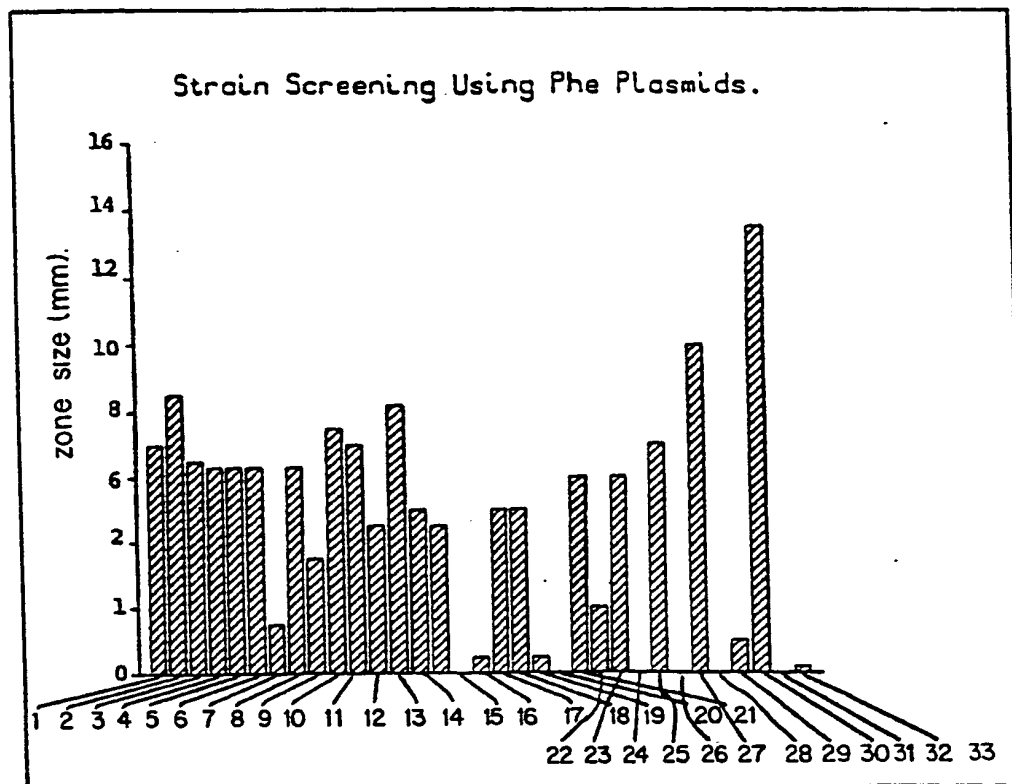


FIG.3

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- | | |
|--------------|-----------------------|
| 1. RTCC9061 | 17. RTCC9085 |
| 2. RTCC9066 | 18. RTCC9086 |
| 3. RTCC9069 | 19. RTCC9087 |
| 4. RTCC9069 | 20. RTCC9088 |
| 5. RTCC9070 | 21. RTCC9089 |
| 6. RTCC9071 | 22. RTCC9091 |
| 7. RTCC9072 | 23. RTCC9092 |
| 8. RTCC9073 | 24. RTCC9093 |
| 9. RTCC9074 | 25. RTCC9094 |
| 10. RTCC9075 | 26. RTCC9095 |
| 11. RTCC9076 | 27. E. Intermedle |
| 12. RTCC9077 | 28. HW77 |
| 13. RTCC9081 | 29. E.eeLL K12 |
| 14. RTCC9082 | 30. HW760 |
| 15. RTCC9083 | 31. S.LyphlewrLum Lt2 |
| 16. RTCC9084 | 32. S. maroeene |

FIG.4

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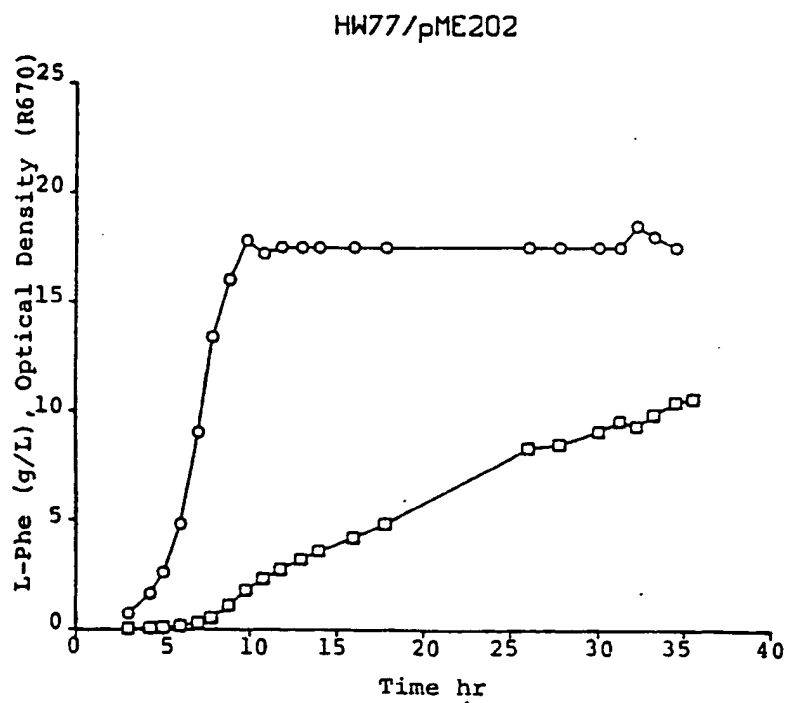
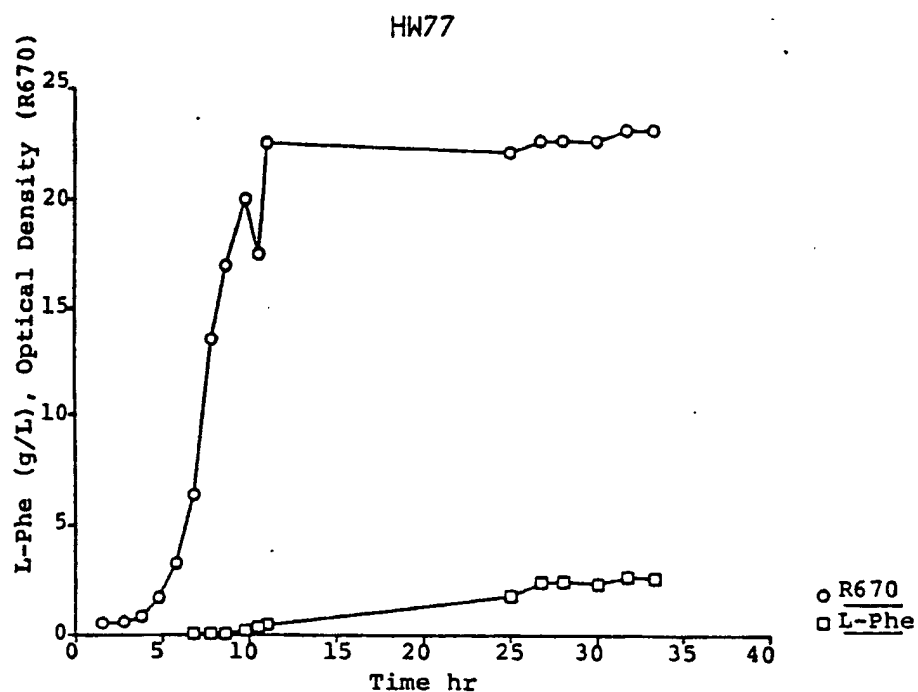


FIG. 5 Comparison of Phenylalanine Production by HW77 and HW77/pME202 During Fermentation.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/01353

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C12P 13/22, C12N 15/00, C12N 1/00		
US: 435/108, 172.3, 317		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/108, 172.3, 253, 317; 536/27 935/27, 40, 56, 60, 72	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
Chemical Abstracts Data Base (CAS) 1967-1986; Biosis Data Base 1969-1986. Keywords: plasmid, vector, clone, phenylalanine, tryptophan, tyrosine, phe, aro, tyr, trp, asp, synthetic operon, transcriptional fusion		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X Y	US, A, 4,371,614 (ANDERSON ET AL) 1 February 1983. See column 4, lines 10-18 column 5, line 67- column 6. line 29, column 7. lines 13-53.	1,4,12,14- 18,24,25, 28 2,3,5-11, 13,19-23, 26,27,29- 34
X Y	EP, A, 0145156 (BIOTECHNICA INT INC) 19 June 1985. See pages 5-7,14-15	1,4,5,6,17. 15,24-26 2,3,7-9,11- 14,16-23, 27-34
Y	EP, A, 0077196 (GENEX CORP) 20 April 1983. See pages 3-5,8,9.	1,4,6,9- 11,15-19, 24-28
<p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ³	Date of Mailing of this International Search Report ³	
28 August 1986	10 SEP 1986	
International Searching Authority ¹	Signature of Authorized Officer ¹⁹	
ISA/US	Karen Maurey Karen Maurey	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ^{1a} with indication, where appropriate, of the relevant passages ^{1c}	Relevant to Claim No ^{1b}
A	FR,A, 2486961 (UNISEARCH LTD) 22 January 1982, see abstract.	16-19
Y	<u>Biochimica et Biophysica Acta</u> , Volume 228. Issued January, 1982 (Amsterdam, Netherlands) Bhosale, et al "Production of chorismate mutase-prephenate dehydrogenase by a strain of <u>Escherichia coli</u> carrying a multicopy <u>tyrA</u> plasmid" pages 6-11. See pages 6 and 10.	1,4,5,7, 8,15-18, 24-27
Y	<u>Gene</u> , Volume 33. Issued May 1985 (Amsterdam, Netherlands) Davies et al "Cloning of <u>aroG</u> , the gene coding for phospho- 2-keto-3-deoxy-heptonate aldolase (phe) in <u>Escherichia coli</u> K-12, and subcloning of the <u>aroG</u> promoter and operator in a promoter detecting plasmid" pages 323-331 see pages 323,324, 329 and 330.	1,4,11,15
A	<u>Gene</u> , volume 36 Issued September 1985 (Amsterdam, Netherlands) Honigman et al "plasmid vectors designed for the analysis of transcription termination signals. pages 131-141, see pages 131-132.	1

